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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Segal et al.
Serial No.:	10/666,886
Filed:	September 19, 2003
Entitled:	Lectin compositions and methods of modulating an immune response to an antigen

Examiner:	Li, E.
Group Art Unit:	1648
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Commissioner for Patents and Trademarks
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Alexandria, VA 22313-1450

RULE 132 DECLARATION OF DR. ANDREW SEGAL

I, Andrew Segal, hereby declare that:

1. I am the sole inventor of the above referenced patent application. I received a B.A. in Biochemical Sciences from Harvard University in 1985, and an M.D. from Boston University School of Medicine in 1990. I conducted postdoctoral research from 1994 to 1996 at the Whitehead Institute of Biomedical Research. I am currently the President and CEO of Genitrix, LLC.

2. I have read the Office Action mailed in the above-referenced patent application on October 11, 2005, and understand that questions have been raised by the Examiner as to whether the disclosure provided by the specification is sufficient to support the breadth of the claimed invention.

3. The experiments described herein were performed under my direction. These experiments were performed *in vivo* in mice. The data described herein show that an engineered cytokine comprising the cytokine GM-CSF fused to an HA1 hemagglutinin domain is mixed with CT26 colon carcinoma cells, K1735 melanoma cells, or B16F10 murine melanoma cells prior to the administration of the resulting composition to a mammal. The results demonstrate that the administration of these compositions in an amount within the ranges taught in the specification, to a mouse inhibits tumor growth and stimulated immune responses compared to the administration of a control composition. The experiments described herein were performed

according to the teachings of the above captioned patent application, and thus support the operability of the vaccine compositions described therein.

The data described herein shows that three different vaccine compositions are demonstrated to be effective in stimulating an immune response in a mammal. As described below, inhibition of tumor development is achieved in a mouse in response to challenge with CT26 murine colon carcinoma cells by administering to the animal a composition comprising CT26 carcinoma cells mixed with GM-CSF-K-HA. In these experiments, GM-CSF-K-HA refers to a fusion protein comprising the cytokine GM-CSF fused via a linker (K) to a heterologous membrane binding domain, influenza hemagglutinin (HA). The experiments below also describe immune stimulation in mice using a composition comprising K1735 melanoma cells mixed with GM-CSF-K-HA, and immune stimulation using a composition comprising CT26 colon carcinoma cells mixed with GM-CSF-K-HA. The experiments below also describe immune stimulation in mice using a composition comprising B16F10 melanoma cells mixed with GM-CSF-HA. The experiments described herein were performed as described in detail in the above-referenced patent application.

Vaccine Composition Comprising CT26 Cells

A vaccine composition was made using CT26 murine colon carcinoma cells. CT26 cells were harvested and washed three times in PBS. Cells were then suspended at 5×10^5 viable cells/ml in PBS, with viability determined by staining an aliquot of cells with Trypan blue. 100 μ l of this suspension was injected into the tail veins of 8-10 week old female BALB/c mice. On day 5 after tumor challenge, mice were immunized with 1×10^6 irradiated CT26 cells subcutaneously in the left inguinal fold. Groups (4 mice each) received either cells alone, cells mixed with 1 μ g soluble recombinant murine GM-CSF (Serologicals Corp.), or cells mixed with 1 μ g of a fusion protein of the invention comprising murine GM-CSF at the N terminus, a (Gly₄Ser)₂ flexible linker, and the HA1 domain of influenza A/PR/8/34 hemagglutinin at the C terminus. The latter composition comprised cell-bound fusion protein as well as non-cell-bound fusion protein, as demonstrated by ELISA assays for GM-CSF.

Mice were sacrificed on day 12, the thoracic cavity opened with dissecting scissors, and the lungs removed *en bloc* by tracheal transection after instillation of India ink. The lungs were

bleached in Feketes solution and tumor nodules were enumerated. The average number of metastases/mouse was as follows:

Cells alone:	37.00
Cells + GM-CSF:	34.75
Cells + fusion protein:	20.25

Thus, administration of a vaccine composition of the invention markedly reduced the average number of lung tumors per mouse, i.e. by 42-46%, even though it was practiced as late as 5 days after the tumors were established.

Vaccine Composition Comprising K1735 Cells

As an allogeneic tumor vaccine model, C57BL/6 mice (haplotype *b*) were immunized with C3H (haplotype *k*)-derived K1735 melanoma cells, followed by challenge with C57BL/6-derived B16F10 melanoma cells. K1735 cells were grown to 70% confluence in DMEM with 10% FBS and penicillin-streptomycin, harvested by trypsinization, and washed 3 times with RPMI 1640. Viability was determined by trypan blue staining of an aliquot and the cells were then resuspended at a concentration of 4×10^6 cells/ml for K1735. One ml aliquots were then dispensed into siliconized microfuge tubes. The cells were incubated with 1 ug GM-CSF-HA (a fusion polypeptide consisting of murine GM-CSF at the N terminus, a (Gly₄Ser)₂ linker, and the HA1 domain of influenza A/PR/8/34) per 10^6 cells for 2 hours at 4° C. An aliquot of the cells was removed for measurement of cell-associated GM-CSF by ELISA. Mean cell-associated GM-CSF across two experiments was approximately 60,000. Cells that were not admixed with any polypeptide and, in one experiment, cells mixed with 1 ug soluble murine GM-CSF (Serologicals Corp.) were prepared in parallel as control vaccines.

The cells were irradiated at 3500 rads from a ¹³⁷Cs source. 8 week-old female C57BL/6 mice were anesthetized by metofane inhalation and vaccinated subcutaneously in the left inguinal fold with or 1×10^6 cells in 0.25 ml RPMI, along with a total of 1 ug GM-CSF-K-HA (including bound and free fusion polypeptide). Each mouse received cells from only one vaccine type. Seven days later, B16F10 cells at 70% confluence were harvested and washed 3 times in HBSS. Viability was determined by trypan blue staining of an aliquot and cells were adjusted in

HBSS to 1×10^5 /ml. The previously vaccinated mice were then challenged subcutaneously behind the neck, under metofane anesthesia, with 0.5 ml of the B16F10 cell suspension.

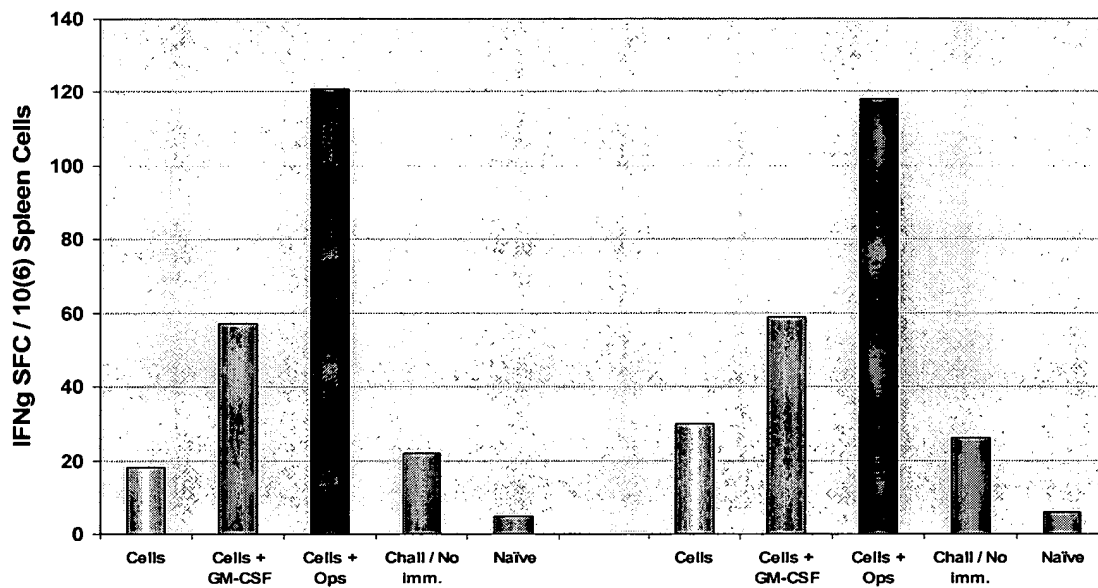
Tumor development was assessed daily by palpation and visual inspection. "Onset" was defined as the first day on which a tumor mass was both palpable and visible. The observer was blinded to the vaccine received by each set of mice to ensure against bias. Mice were sacrificed by CO₂ asphyxiation when tumors become unwieldy. Experiments were terminated 70 days after tumor challenge, as planned in advance.

In pooled results from two experiments, 70 days after challenge, 7/10 mice that had been vaccinated with cells admixed with fusion polypeptide remained tumor-free. In contrast, 10/10 mice that had been vaccinated with cells alone developed tumors, as did 4/5 mice vaccinated with cells admixed with soluble murine GM-CSF.

Vaccine Compositions Comprising B16F10 Cells

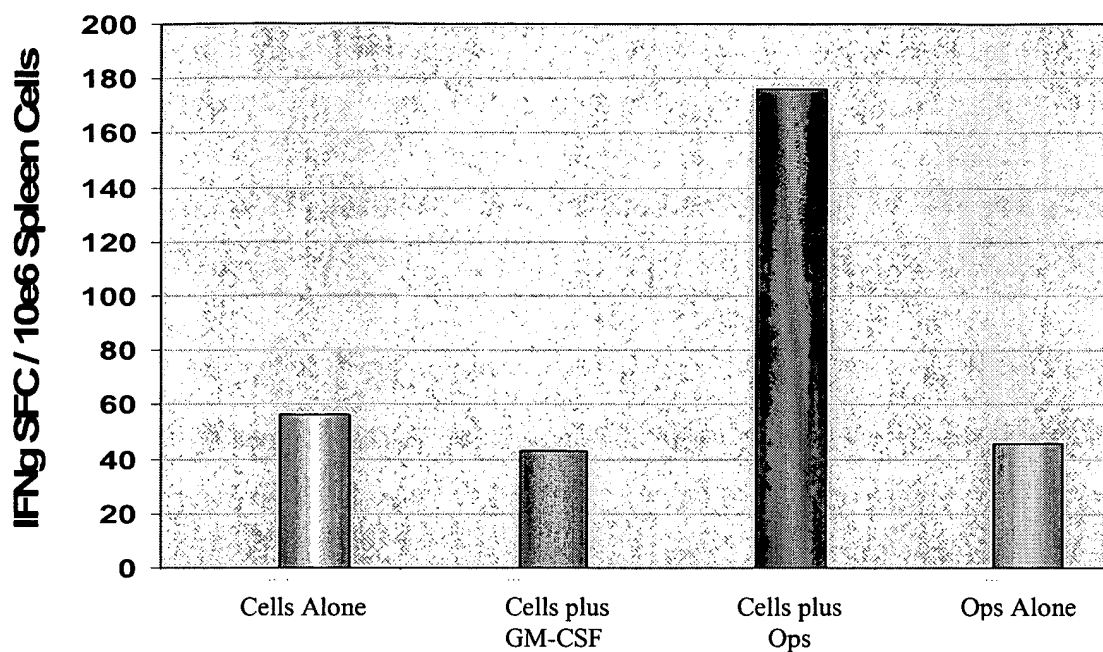
In another experiment, 8-to-10 week old female C57Bl/6 mice (3 animals per group) were challenged once, intravenously, with 5×10^4 wild-type B16F10 murine melanoma cells. Three days later mice were immunized subcutaneously with one of the following: 1×10^6 B16F10 cells alone; 1×10^6 B16F10 cells admixed with 1 ug soluble, recombinant murine GM-CSF; or 1×10^6 B16F10 cells admixed with 1 ug of the GM-CSF-HA fusion protein described hereinabove. Spleen cells were collected 11 days later and the immune response was analyzed by a commercial murine IFN- γ ELISPOT Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's recommendations. Spleen cells from each group were pooled and plated in triplicate wells of 96-well plates at 4×10^5 cells/well in RPMI 1640 (with 10% FBS and pen-strep), mixed with 6×10^5 naïve spleen feeder cells. T cells were stimulated for 24 hr at 37°C in 5% CO₂ with 10 ug/ml of either SVYDFFVWL, an epitope of the B16 antigen TRP-2, or EGSRNQDWL, an epitope of the B16 antigen gp100. Plates were incubated at 37°C, 5% CO₂ for 24 hours and developed according to the manufacturer's protocol. Briefly, the plate was washed four times with provided wash buffer and incubated at 4°C over night with a biotinylated polyclonal antibody specific for mouse IFN-gamma. The next day, following an additional 4 washes, 100 ul of a streptavidin – alkaline phosphatase conjugate were added to each well and incubated at room temperature for 2 hours. After four washes BCIP/NTB chromogen (5-Bromo-

4-Chloro-3' Indolylphosphate p-Toluidine Salt, Nitro Blue Tetrazolium Chloride) were added for 1 hour at room temperature. The plate was finally repeatedly washed with deionized water and dried. Plates were then analyzed electronically (MVS Pacific, LLC, Roseville, MN) using an ELISpot reader system (Carl Zeiss Vision, Germany). Background frequency from challenged but unimmunized mice was subtracted from the counts of each of the groups. The data is shown below, with "Ops" (blue bars) indicating the fusion protein of the invention:



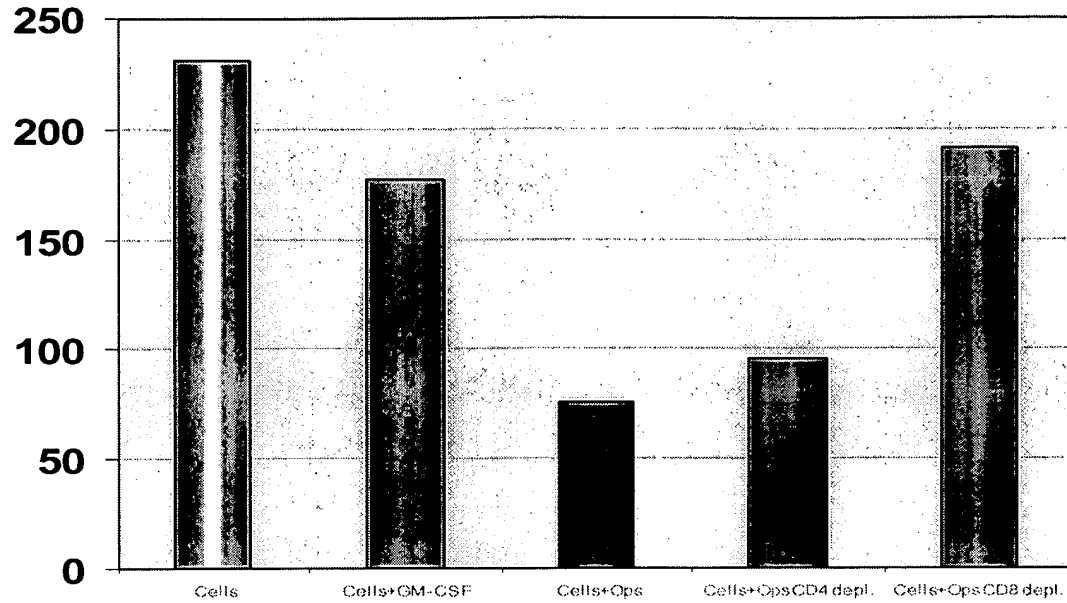
Thus, administration of a vaccine composition of the invention was highly effective at stimulating immune responses to the two structurally distinct antigens TRP-2 and gp100 comprised by the cells of the composition.

Another experiment was performed using methods identical to those above, except that T cells were stimulated in the ELISPOT with lysate from 1×10^5 B16F10 target cells produced by repeated freeze-thawing at -80°C , instead of single peptides. Such lysate comprises all antigens contained in the cells. The data is shown below:



Again, administration of a vaccine composition of the invention was highly effective at stimulating immune responses to antigens comprised by the cells of the composition.

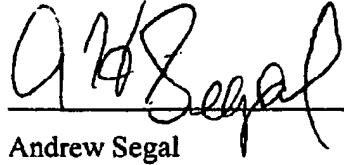
In another experiment, mice were challenged with B16F10 cells and immunized on day 3 as described above. In this instance, additional groups were included in which mice receiving the composition comprising the fusion protein were depleted of either CD4+ or CD8+ T cells by repeated intraperitoneal administration of anti-CD4 or anti-CD8 monoclonal antibody. The data was as follows (“Ops” again denoting the fusion protein):



Thus, the full effect of the therapeutic vaccine comprising the exogenous engineered cytokine was dependent on the presence of T cells, indicating that the composition acted through stimulation of an immune response.

4. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

April 11, 2006
Date


Andrew Segal

A

Identification of Critical Amino Acid Residues in Human and Mouse Granulocyte-Macrophage Colony-stimulating Factor and Their Involvement in Species Specificity*

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Segments critical to the activity of human granulocyte-macrophage colony-stimulating factor (GM-CSF) were identified by scanning deletion analysis and compared with the critical regions previously identified in the homologous mouse GM-CSF protein. Three of the four critical regions thus identified are in equivalent positions in their respective polypeptides, while a fourth critical region of each is uniquely located. To investigate whether unique critical regions are responsible for the observed species specificity of human and mouse GM-CSF, all critical regions were substituted into their opposite homologue. This identified one specific, but different, critical region in each homologue that could not be replaced. Further characterization of the nature of the species specificity of these two proteins was accomplished by the generation of a series of human/mouse GM-CSF hybrids. Each hybrid protein was assayed for specific activity on human- and mouse GM-CSF-dependent cell lines. Significant differences in the specific activity of these hybrids was observed, suggesting that different segments of each molecule interact with their respective receptors. Based on these two approaches, individual amino acids were identified that could provide, at least in part, the interactions between these protein ligands and their respective receptors. These residues are Thr-78 and Met-80 in human GM-CSF and Asp-92, Thr-98, and Asp-102 in mouse GM-CSF.

Granulocyte-macrophage colony stimulating factor (GM-CSF)¹ is a protein hormone that mediates the proliferation, differentiation, and functional activity of neutrophils, macrophages, and eosinophils (2, 3). It is produced by several cell types such as T-lymphocytes and monocytes (4). Both human and mouse GM-CSF have been characterized by cloning, sequencing, and expression of their cDNAs (5, 6). Despite a high level of amino acid homology and similar physical characteristics (7, 8), the two polypeptides are species specific at both the biological and receptor binding levels (6).²

Structure-function studies carried out by us and other laboratories (1, 9-12) have identified a number of residues critical

to the activity of mouse and human GM-CSF. Most of the results support the four α -helical bundle model for human GM-CSF proposed by Parry *et al.* (13). However, none of these studies suggest a basis for the observed species specificity of the two polypeptides.

In the present report we address this question. Two independent approaches were used. First, regions critical to the activity of human GM-CSF were determined and compared with those previously identified in mouse GM-CSF (1). Second, a series of human/mouse hybrid polypeptides were generated and analyzed. The results of these experiments complement each other and indicate that different regions of these structurally homologous proteins provide critical contributions to activity, suggesting a basis for the species specificity of biological activity observed with human and mouse GM-CSF.

MATERIALS AND METHODS

Bacterial Host Strains and Vectors—*Escherichia coli* K12 strain JM101 (14) was used as the host for propagation and maintenance of M13 DNA. CJ236 (15) was used to prepare uracil-DNA for use in site-directed mutagenesis. AB1899 (16) was used as the host for expression of wild-type and mutant human and mouse GM-CSF proteins. Either pIN110mpH3 (17) or pOMPTH3 (a tetracycline-resistant variant of pIN110mpH3) was used as the expression vector for all GM-CSF genes. Elsewhere, we have described the expression of biologically active, mature GM-CSF with this *E. coli* secretory expression system (18). The human GM-CSF coding region was synthetically reconstructed using a series of oligonucleotides. Unique restriction sites were introduced where possible without altering the protein sequence, and preferred *E. coli* codons (19) were selectively used. From this construct, an *Xba*I-*Bam*HI fragment containing the *ompA* leader sequence and the entire human GM-CSF gene was cloned in M13 mp19 (replicative form) and used as the template for site-directed mutagenesis. Construction of the mouse GM-CSF gene and mutagenesis template has been previously described (1).

Mutagenesis, Recombinant DNA, and Sequencing Protocols—*In vivo* recombination of human and mouse GM-CSF was performed as follows: GM-CSF cDNA sequences were oriented in tandem, separated by *Eco*RI and *Sac*I restriction sites, in pIN110mpH3 (17), and were transformed into AB1899. Plasmid DNA was prepared from a bulk culture, cleaved with *Eco*RI and *Sac*I, and retransformed. Individual colonies were screened by restriction enzyme mapping and sequenced using the dideoxynucleotide method (20) with modifications described in the Sequenase[®] (United States Biochemical) protocol.

Site-directed mutagenesis followed the protocol described by Kunzel *et al.* (15). M13 (replicative form) DNA containing correct mutations was cleaved with *Xba*I and *Bam*HI (New England Biolabs) for cloning into pIN110mpH3 or cleaved with *Xba*I and *Eco*RI for cloning into pOMPTH3.

Preparation and Quantitation of Protein Extracts—Protein extracts were prepared as previously described (1). Small aliquots (2-10 μ l) of each sample were assayed for protein concentration in duplicate using a Schleicher & Schuell Minifold II Slot-Blot system. Purified *E. coli*-derived recombinant human or mouse GM-CSF was used on each

* DNAX Research Institute of Molecular and Cellular Biology is supported by the Schering Plough Corporation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom reprint requests should be addressed.

¹ The abbreviation used is: GM-CSF, granulocyte-macrophage colony-stimulating factor.

² A. B. Shanafelt and R. A. Kastelein, unpublished observations.

blot to generate a standard curve for the quantitation of wild-type and mutant protein in each extract. The primary antibody was anti-human GM-CSF peptide hybridoma 2F10 (21) used at a concentration of 1 µg/ml, or anti-mouse GM-CSF peptide hybridoma mg 1.8.2 (22) used at a concentration of 0.2 µg/ml, and secondary antibody was ¹²⁵I-labeled sheep anti-rat IgG (Amersham Corp.) used at 1:1000 dilution. Autoradiograms were scanned by an LKB Ultrascan XL laser densitometer, and the concentration of wild-type and each mutant GM-CSF protein was calculated from the height of each absorption peak in comparison to that generated from a purified GM-CSF standard curve. The error in the calculated concentration of GM-CSF protein by this method was estimated to be approximately 2-fold based on repetitive samples of individual clones of GM-CSF expressed in AB1899.

Proliferation Assays for Human and Mouse GM-CSF Activity—Human GM-CSF deletion protein extracts were assayed using the human GM-CSF-dependent erythroleukemic cell line TF1 (23). GM-CSF protein extracts with substituted critical regions were assayed on both the TF1 cell line and the mouse GM-CSF-dependent myeloid leukemia cell line NFS60. Sample concentrations were adjusted to 108,000 pg/ml and titrated in quadruplicate to 1.8 pg/ml. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay described by Mosmann (24) was used to measure the extent of proliferation, and absorbance values were read with a V_{max} kinetic microplate reader (Molecular Devices, Palo Alto, CA). The concentration of each mutant and wild-type GM-CSF that gave 50% maximum response was determined, and specific relative activity was calculated using the relationship

$$\% \text{ activity} = ([\text{wild-type}]_{1/2} / [\text{mutant}]_{1/2}) \times 100\%,$$

where [wild-type]_{1/2} and [mutant]_{1/2} are the concentrations of wild-type and mutant GM-CSF proteins, respectively, that gave 50% maximum response in the TF1 or NFS60 assays. The specific activity determined varied <10% for a given mutant in multiple assays performed over several months time.

RESULTS

Systematic Introduction of Deletions Throughout Human GM-CSF—The structural and/or functional importance of specific regions within the human GM-CSF polypeptide were examined by the systematic introduction of deletions along the entire length of the molecule. In a manner identical to that described for mouse GM-CSF (1), deletions of three amino acids were introduced every five amino acids on a synthetically generated human GM-CSF DNA sequence by site-directed mutagenesis. The amount of wild-type and mutant polypeptide produced was found to vary from 10–100 µg/ml. Samples were assayed in quadruplicate for their ability to stimulate the proliferation of the human GM-CSF-dependent cell line TF1. Results were expressed as specific activity relative to that of native human GM-CSF produced and assayed under the same conditions in parallel. The sensitivity of the assay was estimated to be 0.01% of wild-type activity.

Definition of Four Regions Critical to the Activity of Human GM-CSF—Fig. 1 illustrates the relative biological activity of 56 deletion proteins with respect to the location of their deletions. This representation displays the relative contribution of each segment of the molecule to the activity of intact human GM-CSF, revealing both critical and noncritical regions. Any mutant protein exhibiting less than 0.01% of wild-type activity was considered inactive, and the amino acid residues in the corresponding deletion mutant were referred to as critical to the activity of human GM-CSF. Four critical regions can be identified comprising ≈36% of the residues. The other 64% of the molecule can tolerate small deletions without complete loss of activity. However, it is evident from Fig. 1 that even the small deletions result in substantial loss of activity.

Comparison of Critical Regions in Human and Mouse GM-CSF—Fig. 2 summarizes the locations of critical regions identified in human GM-CSF compared with those previously

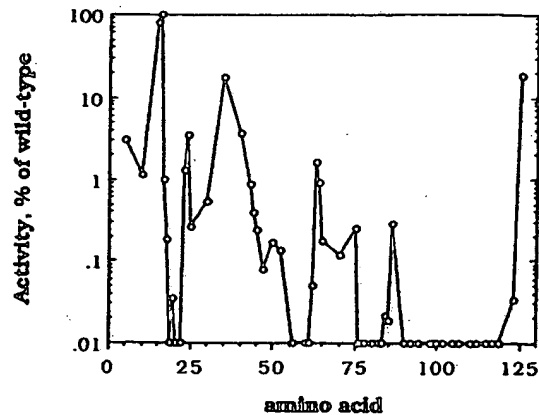


FIG. 1. Relative activity as a function of deletion location. The percent activity relative to wild-type human GM-CSF of each mutant protein in the TF1 assay is plotted as a function of the location of the central amino acid of the respective deletion. Note the logarithmic scale used for % activity. The sensitivity of the assay was estimated to be 0.01% of the wild-type activity; any mutant protein with less than this activity was scored as inactive.



FIG. 2. Comparison of critical regions between human and mouse GM-CSF. The amino acid sequences of human and mouse GM-CSF are shown in parallel, with a three-base insertion in the mouse sequence for alignment purposes. Overall, the two molecules are 54% identical. Critical regions are indicated with bars above (human) and below (mouse) the respective amino acid sequence. Mismatched amino acids between the two homologues within the critical regions are shown in boldface type.

identified in mouse GM-CSF (1). We have adopted a nomenclature for these critical regions in human GM-CSF such that their numerical designation parallels that of those found in mouse GM-CSF (1). It is immediately apparent that the relative positions for critical regions I, III, and IV are comparable in both polypeptides. However, the fourth critical region, labeled IIh in human GM-CSF and IIm in mouse GM-CSF, appears at a unique location in each polypeptide.

Substitution of Critical Regions Has Different Effects on Human and Mouse GM-CSF—The effect of individual critical regions on the activity of human and mouse GM-CSF was examined by substitution of each critical region, including critical regions IIh and IIm, by the corresponding amino acids of the opposite homologue. Substitutions required to change each critical region into the opposite homologue are highlighted in Fig. 2. Mutant proteins were expressed in either pOMPTH3 (human GM-CSF recipient gene) or pNIllom-ph3 (mouse GM-CSF recipient gene), and quantitated as described. All mutants were assayed on both the TF1 and NFS60 cell lines, and relative activity to human GM-CSF (in

TABLE I

Activities of GM-CSF proteins with substituted critical regions

Nomenclature: hu, human; mo, mouse; numerals following indicate critical region substituted. hM2, mouse critical region II_m substituted into the corresponding region of human GM-CSF; mH2, the parallel mutant in mouse GM-CSF. Values are expressed as the % activity relative to native human GM-CSF in the TF1 assay or native mouse GM-CSF in the NFS60 assay (see "Materials and Methods").

Mutant*	TF1	NFS60
% activity		
Mouse critical regions in human GM-CSF		
hu1	>100	≤0.01
hu2	≤0.01	≤0.01
hu3	11	≤0.01
hu4	19	≤0.01
hM2*	>100	≤0.01
hGM-CSF	100	≤0.01
Human critical regions in mouse GM-CSF		
mo1	≤0.01	>100
mo2	≤0.01	>100
mo3	≤0.01	>100
mo4	≤0.01	0.20
mH2*	≤0.01	>100
mGM-CSF	≤0.01	100

* Changes residues not identified as critical in the recipient polypeptide.

the TF1 assay) and mouse GM-CSF (in the NFS60 assay) was determined (Table I).

In human GM-CSF, replacement of critical region I by that of mouse GM-CSF (mutant hu1) had no deleterious effect on activity and substitution of critical regions III and IV had only a moderate effect (mutants hu3 and hu4, respectively) in the TF1 assay. However, substitution of critical region II_h (hu2) resulted in a complete loss of measurable activity (≤0.01%, Table I). In contrast, substitution of critical region II_m (mo2), as well as critical regions I and III (mutants mo1 and mo3, respectively) in mouse GM-CSF by the corresponding sequences in human GM-CSF had no negative effect on activity in the NFS60 assay (Table I), whereas replacement of critical region IV with the corresponding human GM-CSF amino acid residues (mutant mo4) reduced activity to 0.20%.

The inactive human mutant hu2 contains only two amino acid changes, Thr-78 changed to Asn and Met-80 to Thr. Individual substitution of Thr-78 with Asn in human GM-CSF gave a molecule with full activity, while substitution of Met-80 with Thr resulted in a molecule with moderately decreased activity (34%). This demonstrates that individual substitution of these two amino acids in human GM-CSF has little or no effect on activity, but the dual change is sufficient to eliminate the activity of the protein. Mouse GM-CSF mutant mo4 has 14 changes in 26 amino acids, making it difficult to ascribe the loss of activity to any specific amino acid residue. However, analysis of human/mouse GM-CSF hybrid proteins identified mouse residues Asp-92, Thr-98, and Asp-102 as critical for maintaining biological activity (see below).

Substitution of mouse critical region II_m into the corresponding location of human GM-CSF had no negative effect on activity in the TF1 assay, nor did substitution of human critical region II_h into mouse GM-CSF have a negative effect in the NFS60 assay (hM2 and mH2, respectively; Table I). None of the human GM-CSF mutants had any activity in the NFS60 assay, nor did any mouse GM-CSF mutant have any activity in the TF1 assay (Table I).

Generation of Hybrid GM-CSF Polypeptides—A second,

independent approach to identify regions and/or residues essential for biological activity uses the species specific character of human and mouse GM-CSF. Hybrid proteins were generated by either *in vivo* recombination or site-directed mutagenesis (15) on appropriate templates. The naming of the hybrid proteins follows the convention that either an H (human) or M (mouse) designates the species of the N-terminal portion of the hybrid, and the following numbers indicate the amino acids at which crossover occurs between the species. For example, H 6/7 contains the first six amino acids of mature human GM-CSF (Ala = 1) and amino acids 7–124 of mature mouse GM-CSF (Ala = 1).

The altered GM-CSF coding regions were expressed and quantitated as described. All mutant polypeptides were assayed on both the TF1 and NFS60 cell lines and relative activity to human GM-CSF (in the TF1 assay) or mouse GM-CSF (in the NFS60 assay) was determined (Table II).

Nonsymmetrical Effects of N- and C-terminal Substitution on Biological Activity—The effects of substitution on the ends of the two GM-CSF proteins are shown graphically in Fig. 3, where the relative activity in the TF1 or NFS60 assay is plotted against the amino acid sequence crossover point of each hybrid.

The effect of N-terminal substitution (Fig. 3A) in human GM-CSF is described by N-terminal mouse/C-terminal human hybrids assayed in the TF1 assay, and on mouse GM-CSF by N-terminal human/C-terminal mouse hybrids assayed in the NFS60 assay. In human GM-CSF, 35% can be substituted to yield a fully active molecule (M 43/43, Table II). A further substitution of hybrid M 43/43 by only one amino acid (M 45/45, additional change of human Ile-43 to Phe) causes a 500-fold decrease in activity. Mouse GM-CSF, on the other hand, loses activity after substitution of only 17 N-terminal amino acids. The single additional amino acid change in the N terminus of Glu-17 to Asn between H 16/17 and H 19/20 in mouse GM-CSF resulted in a 5,000-fold decrease in activity (Table II).

A direct comparison of C-terminal substitutions is shown in Fig. 3B. Here, N-terminal human/C-terminal mouse hybrids in the TF1 assay describe C-terminal substitution of human GM-CSF, and N-terminal mouse/C-terminal human hybrids in the NFS60 assay show the effects of C-terminal substitution in mouse GM-CSF. Human GM-CSF loses about 99% of its activity after substitution of only four amino acids (H 121/119, 0.79%); yet in mouse GM-CSF, the C terminus is unaffected by substitution of the terminal 22 amino acids. Not until the additional Asp-102 to Ser change occurs between M 103/107 and M 101/105 is a loss of activity observed. Two other similar activity changes are seen in this region resulting from net single amino acid changes: Thr-98 to Ile (M 97/101) and Asp-92 to Ser (M 91/95).

Single Amino Acid Substitutions of GM-CSF—In a number of cases large changes in activity were observed between hybrids that only differ by one amino acid. To examine these potentially important amino acid positions independent of the hybrid context single amino acid substitution of wild-type human and mouse GM-CSF were made.

Human GM-CSF—Large changes in activity of adjacent hybrids occur at two places, between M 43/43 and M 45/45, and H 122/120 and H 121/119 (Table II). The net amino acid changes were, respectively, Ile-43 to Phe and Trp-122 to Lys. These individual substitutions were generated by site-directed mutagenesis and assayed in the TF1 assay.

The results are shown in Table III, where the activity of each single amino acid mutant is shown in boldface type adjacent to the activity of its corresponding hybrid. Changes

TABLE II
GM-CSF hybrid proteins and their activity
on human and mouse cell lines

Hybrid ^a	% of parent polypeptide ^b	Activity, % of wild-type ^c	
		TF1	NFS60
H 6/7 ^d	4.7	<0.01	180
H 16/17 ^d	12.6	<0.01	50.0
H 19/20	15.0	<0.01	<0.01
H 22/23	17.3	<0.01	<0.01
H 35/36	27.6	<0.01	<0.01
H 44/46	34.6	<0.01	<0.01
H 49/50	38.6	<0.01	<0.01
H 54/52	42.5	<0.01	<0.01
H 59/57 ^d	46.5	<0.01	<0.01
H 68/66 ^d	53.5	0.08	<0.01
H 75/73 ^d	59.1	0.06	<0.01
H 77/75 ^d	60.6	0.04	<0.01
H 94/92 ^d	74.0	0.17	<0.01
H 119/117 ^d	93.7	1.2	<0.01
H 121/119	95.3	0.79	<0.01
H 122/120	96.1	8.6	<0.01
H 124/122	97.6	40	<0.01
H 126/124	99.2	73	<0.01
hGM-CSF	100.0	100	<0.01
M 6/7 ^d	4.8	5.6	<0.01
M 22/23	17.7	12	<0.01
M 35/36	28.2	43	0.38
M 43/43 ^d	34.7	210	0.13
M 45/45	36.3	0.38	<0.01
M 46/46	37.1	0.18	<0.01
M 47/47	37.9	0.04	<0.01
M 48/48	38.7	1.1	1.3
M 49/50	39.5	0.06	0.64
M 51/55	41.1	0.17	6.6
M 56/60 ^d	45.2	0.06	0.29
M 65/69 ^d	52.4	<0.01	0.04
M 72/76 ^d	58.1	<0.01	0.03
M 74/78 ^d	59.7	<0.01	0.04
M 81/85 ^d	65.3	<0.01	0.03
M 91/95 ^d	73.4	<0.01	0.03
M 93/97	75.0	<0.01	0.90
M 96/100	77.4	<0.01	1.0
M 97/101	78.2	<0.01	1.4
M 99/103	79.8	<0.01	14
M 100/104	80.6	<0.01	17
M 101/105	81.5	<0.01	6.6
M 103/107	83.1	<0.01	110
M 116/120	93.5	<0.01	100
mGM-CSF	100.0	<0.01	100

^a The nomenclature follows the convention that either an H (human) or M (mouse) designates the species of the N-terminal portion of the hybrid protein, and the following numbers indicate the amino acids at which crossover occurs between the species.

^b The parent polypeptide is defined as that comprising the N-terminal of the hybrid. The percent composition reflects the number of amino acid residues of the N-terminal species contained in the hybrid divided by the total number of residues in the mature wild-type GM-CSF protein.

^c Activity is expressed as the % activity relative to native GM-CSF in the TF1 or NFS60 assay (see "Materials and Methods").

^d These hybrids were generated by *in vivo* recombination as described in "Materials and Methods." All others were made using site-directed mutagenesis on appropriate templates (15).

to Ile-43 and Trp-122 yield proteins with activities similar to the appropriate hybrid, suggesting that Ile-43 and Trp-122 are integral in their contribution to the activity of human GM-CSF.

Mouse GM-CSF—The greatest variations in activity occur between hybrids H 16/17 and H 19/20, M 48/48 and M 47/47, and at the C terminus between M 103/107 and M 101/105, M 99/103, and M 97/101, and M 93/97 and M 91/95, for

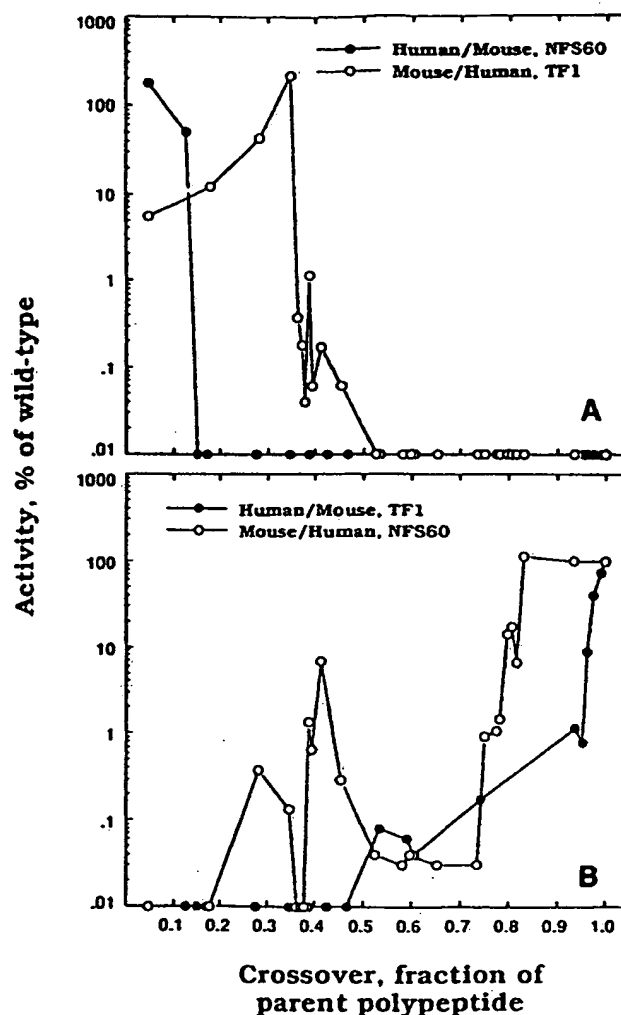


FIG. 3. Differences in the N- and C-terminal responses between human and mouse GM-CSF. Activity of the hybrid proteins were measured in the TF1 (human) or NFS60 (mouse) assays. Activity is presented as a function of the fraction of the hybrid that is made up of the N-terminal homologue. Human/mouse hybrids (●) and mouse/human hybrids (○) are grouped cross-assay to compare parallel hybrids. In panel A, effects of substitution on activity of the N-terminal regions of both proteins are shown. Panel B displays the effects of C-terminal substitution. In both cases, large variations in the response pattern between the two polypeptides are evident.

a combined decrease from 113 to 0.03% activity (Table II). The net amino acid changes were, respectively, Glu-17 to Gln, Lys-48 to Asp, Asp-92 to Ser, Thr-98 to Ile, and Asp-102 to Ser. The activities of the mutants were measured in the NFS60 assay and are displayed in boldface type next to the activity of the corresponding hybrid in Table III.

Both mE17N and mK48D have greater than full activity, indicating that a combined effect of the hybrid substitutions was responsible for the loss in activity in the corresponding hybrid molecules. The single change in mD92S has no significant effect on activity, while mT98I and mD102S both give about a 10-fold reduction in activity. Since these 3 residues fall at or in the only critical region in mouse GM-CSF that was sensitive to substitution (critical region IV) we examined the combined effect of these three mutations on the activity of mouse GM-CSF. Two double mutants, mDT:SI (Asp-92 and Thr-98 to Ser and Ile) and mTD:IS (Thr-98 and Asp-102 to Ile and Ser) still had about 10% activity (Table III).

TABLE III
Comparison of individual amino acid mutants
with corresponding hybrid GM-CSF protein

Mutant GM-CSF	Corresponding hybrid	Activities, % of wild-type
Human GM-CSF ^a		
hI43F	M 45/45	0.50/0.38
hW122K	H 121/119	1.3/0.79
Mouse GM-CSF ^b		
mE17N	H 19/20	340/0.01
mK48D	M 47/47	580/0.01
mD92S	M 91/95	86/0.03
mT98I	M 97/101	8.5/1.4
mD102S	M 101/105	9.2/6.6
mDT:SI ^c	M 91/95	12/0.03
mTD:IS ^d	M 97/101	5.5/1.4
mDTD:SIS ^e	M 91/95	0.02/0.03

^a Activities measured in the TF1 assay.

^b Activities measured in the NSF60 assay.

^c mDT:SI = D92S + T98I in mouse GM-CSF.

^d mTD:IS = T98I + D102S in mouse GM-CSF.

^e mDTD:SIS = D92S + T98I + D102S in mouse GM-CSF.

However, the combined change of all 3 residues produces a molecule with minimal activity (mDTD:SIS, Table III). This triple mutant has activity equal to its corresponding hybrid (M 91/95), indicating that these 3 amino acid residues together act significantly in the activity of mouse GM-CSF. Substitution of the corresponding amino acid residues in human GM-CSF, Ser-95, Ile-101, and Ser-105 to their mouse GM-CSF counterparts had no effect on activity in the TF1 assay.

DISCUSSION

A striking feature of human and mouse GM-CSF is their absolute species specificity despite a high degree of sequence homology. Both molecules are physically similar polypeptides (8) with identical disulfide structures (7), and both are suggested to have comparable structures using predictive algorithms (13, 25). The considerable homology between the two polypeptides suggests that differences exposed by similar forms of analysis are contributing factors to the uniqueness of each molecule. Two independent approaches were used to test this hypothesis. First, we determined and compared regions critical to the biological activity of human GM-CSF with those previously identified in mouse GM-CSF (1). Second, we have generated and analyzed a series of human/mouse hybrid proteins.

Fig. 2 graphically compares the critical regions found in human and mouse GM-CSF. The structural homology between the two polypeptides is reflected in the corresponding locations of three out of the four critical regions (regions I, III, and IV). Only critical regions IIh in human GM-CSF and IIIm in mouse GM-CSF occupy unique positions in their respective polypeptides. We anticipated that the difference in location of these critical regions could account (at least to some extent) for the species specific character of GM-CSF. To test this hypothesis we substituted not only the uniquely located critical regions IIh and IIIm, but also the common critical regions in both molecules with residues of the opposite species. Both molecules are generally tolerant to substitution with minor or no decrease in activity (Table I). However, in each molecule one specific, but different, critical region is sensitive to substitution. In human GM-CSF, substitution of critical region IIh eliminates activity, while a similar decrease was observed when critical region IV was substituted in mouse GM-CSF.

Critical Amino Acid Residues in Human GM-CSF—Sensitivity of human critical region IIh to substitution with mouse residues supports the hypothesis that the residues comprising this region, amino acids at positions 77–82, contribute to the species-specific character of GM-CSF. The present study identifies specifically residues Thr-78 and Met-80 in this region as important residues. Although individual substitution of these residues does not affect the activity significantly, substitution of both amino acids to the corresponding mouse residues (mutant hu2) completely eliminates it. This double mutant also is unable to compete native human GM-CSF in binding assays on TF1 cells (data not shown).

Two other lines of evidence support the notion that residues 77–82 form species-specific receptor contacts. Brown *et al.* (11) have mapped the epitopes of a pair of blocking monoclonal antibodies to residues 77–94. Secondly, in a previously published study involving hybrid GM-CSF molecules, Kaushansky and co-workers (10) identified residues 78–94 as critical for functional activity. Our hybrid GM-CSF data do not specifically identify human GM-CSF residues 77–82 as critical. An explanation for this could be the low activity of the two hybrids that cover this region (H 77/75 and H 94/92, Table II).

Kaushansky *et al.* (10) also stipulate that amino acid residues 21–31 are critical for activity of human GM-CSF. Our data do not support this conclusion. Substitution of the first 43 residues of human GM-CSF resulted in a protein with full activity (Table II, M 43/43). This indicates that human GM-CSF can sustain extensive substitution in this region, although it is intolerant to small deletions (Fig. 1) and specific substitutions (*e.g.* residues 20 and 21, Ref. 26). Apparently, the structure of human GM-CSF can be disrupted by mutations in the N-terminal 43 amino acids, but it can be maintained by complete substitution with mouse residues. Consequently, this region cannot be involved in the species specificity observed for human GM-CSF.

In the hybrid analysis we observed a sharp reduction in activity across human GM-CSF residues 122–126. This decrease in activity was not observed by either Clark-Lewis *et al.* (9), using synthesized human GM-CSF peptides, or by Kaushansky *et al.* (10). However, neither Clark-Lewis *et al.* nor Kaushansky *et al.* accurately quantitated the amount of specific mutant protein examined in their assays, and thus may explain why these two groups did not see this effect. Deletions in the C-terminal portion of human GM-CSF also caused significant losses of activity (Fig. 1). We have purified to near homogeneity, quantitated, and assayed C-terminal deletion mutants of both human and mouse GM-CSF which lack the final 6 amino acid residues of each protein (data not shown). Consistent with our hybrid data, the human deletion mutant has 3.0% of wild-type activity (compare with H 121/119, 0.79%, and hW122K, 1.3% activity), and the mouse deletion mutant has 68% of wild-type activity (compare with M 116/120, 100% activity). The sensitivity of these residues in human GM-CSF to both substitution (specifically Trp-122) and deletion alludes to the possibility that they have a material role in the activity of this protein, whereas this region appears to be of no importance in mouse GM-CSF.

A specific role for the C terminus of human GM-CSF is further supported by the work of Seelig *et al.* (12). These authors show that purified rabbit IgG raised against a C-terminal peptide of human GM-CSF (residues 110–127) blocks the activity of human GM-CSF. Anti-idiotypic antibodies generated against these IgG proteins did not bind to human GM-CSF or the peptide, yet specifically blocked human GM-CSF activity and inhibited ¹²⁵I-human GM-CSF

receptor binding. These data support the conclusion that a portion of these residues is close to or in direct contact with the receptor.

Two additional amino acid residues in human GM-CSF were identified as critical for activity in the native polypeptide, Ile-43 and Trp-122, with Trp-122 likely to be at or near the receptor-binding site. At present we have no data to suggest that Ile-43 has a functional role. This residue may be important for maintaining structural integrity.

Critical Amino Acid Residues in Mouse GM-CSF.—Tolerance of the unique mouse critical region II_m to substitution with human residues indicates that these residues do not contribute to the species-specific character of mouse GM-CSF. This is true for all critical regions in human and mouse GM-CSF that retain significant activity upon substitution with residues of the other species. It is likely that these regions are critical (as defined by deletion analysis) because they fulfill a structural requirement of the molecule. Since mouse, but not human, critical region IV is sensitive to substitution, it suggests that this region provides some species-specific feature to mouse GM-CSF. The analysis of hybrid mouse/human GM-CSF molecules across this region identifies 3 residues (Asp-92, Thr-98, and Asp-102) as significant for mouse GM-CSF activity. Just as was observed for residues Thr-78 and Met-80 of the unique human GM-CSF critical region II_h, individual substitution of the 3 mouse GM-CSF residues had no dramatic effect on activity. Substitution of all 3 residues by the corresponding human GM-CSF residues rendered the resulting mutant mouse GM-CSF polypeptide virtually inactive, suggesting that some cooperative effect between these 3 residues is occurring. This effect is unique to mouse GM-CSF, since neither substitution of human GM-CSF critical region IV nor simultaneous substitution of the comparable human GM-CSF residues (residues Ser-95, Ile-101, and Ser-105) had any profound effect on activity.

In mouse GM-CSF, two additional large changes in activity (>100-fold) occurred as an apparent consequence of single amino acid substitutions. However, individual substitution of these residues, Glu-17 and Lys-48, had no deleterious effect on activity, reflecting that some cumulative effect of substitutions in the corresponding hybrid proteins was necessary to affect activity.

What Defines Species Specificity in Human and Mouse GM-CSF?—By analyzing human and mouse GM-CSF using similar approaches, significant differences were exposed despite the overall physical similarities of both molecules. First of all, by substituting critical regions it was found that each molecule contains one specific, but different, critical region that is sensitive to substitution. The unique character of each polypeptide was further reflected by their differential sensitivity to N- and C-terminal substitution with residues of the other species. Thus, the interactions of the two ligands with their respective receptors appear to involve different regions of the ligands. For human GM-CSF, the evidence available suggests that residues in critical region II_h, in particular Thr-78 and Met-80, provide receptor contacts. The C terminus of human GM-CSF, and specifically residue Trp-122, is also either part of or in close proximity to the human GM-CSF receptor-binding domain. In mouse GM-CSF, it is possible that a combination of residues Asp-92, Thr-98, and Asp-102 provides species-specific receptor contacts. A second important region in mouse GM-CSF is located around residues 17–22. At present, no data are available related to whether these residues form receptor interactions or whether they fulfill a critical structural role; however, deletion mutants and N-terminal human/C-terminal mouse GM-CSF hybrids have identified

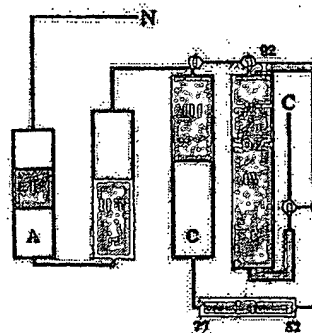


FIG. 4. Critical regions superimposed on proposed secondary structure of human and mouse GM-CSF. Secondary structure of the proposed four-helix bundle model for GM-CSF is adapted from Parry *et al.* (13), and is drawn approximately to scale. Alphabetically labeled helices are indicated by rectangular boxes. Critical regions, indicated by roman numerals, are superimposed as shaded areas along the linear sequence with the unique critical regions II_h and II_m heavily outlined (some superposition of critical regions occurs, e.g. IV). Cysteines are numbered sequentially and indicated by black circles; disulfide bridges are shown by dotted lines. The parallel nature of helices C and D as required by the disulfide pairing is also represented. Mouse GM-CSF residues Asp-92, Thr-98, and Asp-102 are indicated by heavy lines.

this region as absolutely required for mouse GM-CSF activity. The full activity of human GM-CSF hybrids with a mouse GM-CSF N terminus of up to 45 residues excludes a contribution of this region to specificity of human GM-CSF.

The differences between human and mouse GM-CSF described above may form the basis for the species specificity observed between these two molecules. We have superimposed these data on a four α -helical bundle model for human GM-CSF proposed by Parry *et al.* (13) (Fig. 4). Strikingly, the shared critical regions between human and mouse GM-CSF, namely I, III, and IV, coincide precisely with regions predicted to be α -helical. This similarity between human and mouse GM-CSF suggests that these helices play an important role in maintaining protein integrity and bioactivity. In human GM-CSF, critical region II_h forms the beginning of the C-D loop. If loops and coils at the end of helices are the most likely sites of receptor interactions (as proposed by Parry *et al.* (13)), it is possible that the residues defining critical region II_h in human GM-CSF form direct receptor contacts; both deletion and substitution eliminate activity. The C-terminal residues 122–127 are potentially in close proximity to the residues of critical region II_h and could form part of the receptor-binding domain. The critical mouse GM-CSF residues Asp-92, Thr-98, and Asp-102 are located in the N-terminal portion of mouse critical region IV, coinciding with helix D in the Parry model (Fig. 4). Structural studies of mouse GM-CSF (27) using proline substitutions suggest that the N-terminal portion of critical region IV, predicted to be α -helical, is only loosely defined by this structural motif. Thus, it is possible that the N-terminal portion of critical region IV, and specifically residues Asp-92, Thr-98, and Asp-102, form a loop-like structure that is free to interact with the mouse GM-CSF receptor.

If the Parry *et al.* (13) model is correct, critical region II_h and the N-terminal portion of critical region IV lie on opposite poles of the molecule (Fig. 4). Indeed, if these are the sites of direct receptor interactions for their respective proteins, the species specificity of human and mouse GM-CSF can be explained on this basis alone. Confirmation of this conclusion is dependent upon further mutational analysis of the residues involved and, eventually, three-dimensional structural information on each polypeptide.

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REFERENCES

- Shanafelt, A. B., and Kastelein, R. A. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 4872-4876
- Metcalf, D. (1986) *Blood* **67**, 257-267
- Gough, N. M., and Nicola, N. A. (1989) in *Colony-Stimulating Factors: Molecular and Cellular Biology* (Dexter, T. M., Garland, J. M., and Testa, N. G., eds) pp. 111-153, Marcel Dekker, New York
- Metcalf, D. (1985) *Science* **229**, 16-22
- Gough, N. M., Metcalf, D., Gough, J., Grail, D., and Dunn, A. R. (1985) *EMBO J.* **4**, 645-653
- Lee, F., Yokota, T., Otsuka, T., Gemmell, L., Larson, N., Luh, J., Arai, K., and Rennick, D. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 4360-4364
- Schrimsher, J. L., Rose, K., Simona, M. G., and Wingfield, P. (1987) *Biochem. J.* **247**, 195-199
- Wingfield, P., Graber, P., Moonen, P., Craig, S., and Pain, R. H. (1988) *Eur. J. Biochem.* **173**, 65-72
- Clark-Lewis, I., Lopez, A. F., To, L. B., Vadas, M. A., Schrader, J. W., Hood, L. E., and Kent, S. B. (1988) *J. Immunol.* **141**, 881-889
- Kaushansky, K., Shoemaker, S. G., Alfaro, S., and Brown, C. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 1213-1217
- Brown, C. B., Hart, C. E., Curtis, D. M., Bailey, M. C., and Kaushansky, K. (1990) *J. Immunol.* **144**, 2184-2189
- Seelig, G. F., Prosser, W. W., Scheffler, J. E., Nagabhushan, T. L., and Trotta, P. P. (1990) *J. Cell. Biochem.* **14C** (suppl.), 1246
- Parry, D. A. D., Minasian, E., and Leach, S. J. (1988) *J. Mol. Recogn.* **1**, 107-110
- Messing, J. (1983) *Methods Enzymol.* **101**, 20-78
- Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol.* **154**, 367-382
- Howard-Flanders, P., Simson, E., and Theriot, L. (1964) *Genetics* **49**, 237-246
- Lundell, D., Greenberg, R., Alroy, Y., Condon, R., Fossetta, J. D., Gewain, K., Kastelein, R., Lunn, C. A., Reim, R., Shah, C., Van Kimmenade, A., and Narula, S. K. (1990) *J. Indust. Microbiol.* **5**, 215-228
- Greenberg, R., Lundell, D., Alroy, Y., Bonitz, S., Condon, R., Fossetta, J., Frommer, B., Gewain, K., Katz, M., Leibowitz, P. J., Narula, S. K., Kastelein, R., and Van Kimmenade, A. (1988) *Curr. Microb.* **17**, 321-332
- De Boer, H. A., and Kastelein, R. (1986) in *Maximizing Gene Expression* (Resnikoff, W., and Gold, L., eds) pp. 225-283, Butterworth, Totaham
- Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463-5467
- Abrams, J. S., and Laquoi-Ledger, C. (1987) *J. Biol. Response Modif.* **6**, 222
- Miyajima, A., Otsu, K., Schreurs, J., Bond, M. W., Abrams, J. S., and Arai, K. (1986) *EMBO J.* **5**, 1193-1197
- Kitamura, T., Tojo, A., Kuwaki, T., Chiba, S., Miyazono, K., Urabe, A., and Takaku, F. (1989) *Blood* **73**, 375-380
- Mosmann, T. (1983) *J. Immunol. Methods* **65**, 55-63
- Chou, P. Y., and Fasman, G. D. (1978) *Annu. Rev. Biochem.* **47**, 251-276
- Lopez, A. F., Shannon, M. F., To, L. B., and Vadas, M. A. (1989) *Leuk. Res.* **13** (Suppl. 1), 10
- Altmann, S. W., Johnson, G. D., and Prystowsky, M. B. (1991) *J. Biol. Chem.*, in press

Identification of critical regions in mouse granulocyte-macrophage colony-stimulating factor by scanning-deletion analysis

(expression/structure-function/mutagenesis)

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ABSTRACT Structure-function relationships for mouse granulocyte-macrophage colony-stimulating factor were examined by generating a series of small deletions scanning the entire length of the molecule. Deletions of three amino acids were introduced at intervals of five amino acids by site-directed mutagenesis of the mature mouse granulocyte-macrophage colony-stimulating factor gene. The mutant proteins were expressed in *Escherichia coli* and assayed for biological activity. This procedure identified four regions critical to activity. These critical regions were further delineated by additional three-amino acid deletion mutants. Larger deletions at each terminus were also made, as well as changes of specific amino acid residues. The four critical regions span amino acid residues 18-22, 34-41, 52-61, and 94-115. The disulfide bridge between Cys-51 and Cys-93 was also shown to be essential for activity, whereas that between Cys-85 and Cys-118 could be removed without loss of activity. The possible structural and/or functional roles of the critical regions are discussed.

The proliferation and differentiation of granulocytes and macrophages from progenitor bone marrow cells is mediated by a group of protein hormones known as the colony-stimulating factors (1). Four distinct colony-stimulating factors have been identified in both mouse and human; one of these is granulocyte-macrophage colony-stimulating factor (GM-CSF). It is produced by various cell types, such as T lymphocytes and monocytes (2), and has multiple activities *in vitro*. It not only stimulates the formation of granulocytes and macrophages from bone marrow progenitor cells (1) but also can activate mature eosinophils, neutrophils, and macrophages (3).

Both mouse (m) and human (h) GM-CSF have been characterized by cloning, sequencing, and expression (4, 5). The mGM-CSF polypeptide (124 residues; M_r , 14,346) is 54% identical to the hGM-CSF polypeptide (127 residues; M_r , 14,650). Despite the high degree of homology, the two polypeptides are species specific (5).

Limited information is available on structure-function relationships for GM-CSF. Proteolytic digestion of both proteins identified two disulfide bonds, linking the first and third, and second and fourth cysteine residues (6). Using chemically synthesized hGM-CSF protein fragments and analogs, Clark-Lewis *et al.* (7) identified residues 1-13 and residues 122-127 as being not critical and residues 14-25 as being critical for biological activity. Mutagenesis of mGM-CSF by Gough *et al.* (8) revealed that residues 11-15, 24-37, 47-49, and 81-89 are obligatory for function. Physical and biochemical studies indicate that both the murine and human proteins have similar secondary structures containing α -helical and β -sheet segments (9).

In this report we describe an analysis of a set of deletion mutants that together span the entire length of mGM-CSF. Characterization of 42 deletion proteins, expressed in *Escherichia coli*, has allowed us to identify and delineate four regions within mGM-CSF that are critical to the activity of the protein. Additional deletion and substitution mutants further define the importance of these and other regions of the molecule.

MATERIALS AND METHODS

Bacterial Host Strains and Vectors. The *E. coli* K12 strain JM101 (10) was used as host for the propagation and maintenance of M13 DNA. CJ236 (11) was used to prepare uracil-DNA for use in site-directed mutagenesis. AB1899 (12) was used as the host for expression of wild-type and mutant mGM-CSF. pIN11IompA2 (13) was used as the expression vector for all mGM-CSF genes. Elsewhere, we have described the expression of biologically active, mature GM-CSF with this *E. coli* secretory expression system (14). The mGM-CSF coding region was synthetically reconstructed using a series of oligonucleotides. Unique restriction sites were introduced where possible without altering the protein sequence and high-bias *E. coli* codons (15) were preferentially used. From this construct, an *Xba*I-*Bam*HI fragment containing the *ompA* leader sequence and the entire mGM-CSF gene was cloned in M13mp19 (replicative form) and used as the template for site-directed mutagenesis.

Mutagenesis, Recombinant DNA, and Sequencing Protocols. Site-directed mutagenesis followed the protocol described by Kunkel *et al.* (11). Oligonucleotides, 20-28 nucleotides long, corresponding to mGM-CSF sequences incorporating the desired deletion or mutation were made complementary to the template DNA and used as primers in the mutagenesis reactions. Three (to five) plaques from each reaction were sequenced using the dideoxynucleotide method (16) with modifications described in the Sequenase (United States Biochemical) protocol. Where possible, the entire GM-CSF coding sequence was examined.

M13 (replicative form) DNA containing correct mutations was cleaved with *Xba*I and *Bam*HI (New England Biolabs). The digested M13 (replicative form) was combined in a 40- μ l ligation mixture containing 0.2 μ g of pIN11IompA2 (cleaved with *Xba*I and *Bam*HI) and incubated at 12°C for 4 hr. The ligation mixture was used to transform JM101 cells, and selection was made on Luria broth (LB) plates containing ampicillin at 50 μ g/ml. Correct clones were transformed into AB1899 for protein expression.

Gel Electrophoresis and Immunoblotting. SDS/polyacrylamide gel electrophoresis (SDS/PAGE) was performed as described by Laemmli (17). Immunoblotting was performed essentially as described by Towbin *et al.* (18), except that

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Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; m, mouse; h, human.

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Table 1. Activity and alterations of mutant mGM-CSF proteins

Mutant protein [†]	Alteration [‡]	% activity [§]
V1	4-6	343
V2	9-11	789
V3	14-16	70.3
V4	19-21	<0.01
V5	24-26	0.8
V6	29-31	4.6
V7	34-36	<0.01
V8	39-41	<0.01
V9	44-46	1.9
V10	48-50	0.3
V11	54-56	<0.01
V12	59-61	<0.01
V13	64-66	572
V14	69-71	5.7
V15	74-76	1.1
V16	79-81	3.0
V17	83-84;86-87	9.9
V18	89-91	0.07
V19	94-96	<0.01
V20	99-101	<0.01
V21	104-106	<0.01
V22	109-111	<0.01
V23	114-116	<0.01
V24	119-121	167
V25	15-17	5.6
V26	16-18	<0.01
V27	17-19	<0.01
V28	23-25	0.7
V29	31-33	3.7
V30	42-44	0.1
V31	52-54	<0.01
V32	61-63	<0.01
V33	62-64	0.03
V34	63-65	0.03
V35	90-92	0.02
V36	117-119	1.5
V37	96-98	<0.01
V38	102-104	<0.01
V39	107-109	<0.01
V40	112-114	<0.01
V41	21-23	<0.01
V42	22-24	<0.01
VN19	2-19	<0.01
VC94	94-124,	
	Cys-85 → Ser	<0.01
VC116	116-124	94.1
CI118	Cys-118 → Stop	158
K119	Lys-119 → Stop	968
C51S	Cys-51 → Ser	<0.01
C85S	Cys-85 → Ser	15.4
C93S	Cys-93 → Ser	<0.01
CI118S	Cys-118 → Ser	6.1
C51-93S	Cys-51 → Ser	
	Cys-93 → Ser	<0.01
C85:118S	Cys-85 → Ser,	
	Cys-118 → Ser	129
mGM-CSF	Wild-type	100

[†]V1 to V24 were the first series of deletion mutants tested.

[‡]Numbers indicate residues deleted in deletion mutants or residue changes.

[§]Activity is relative to that of wild-type mGM-CSF.

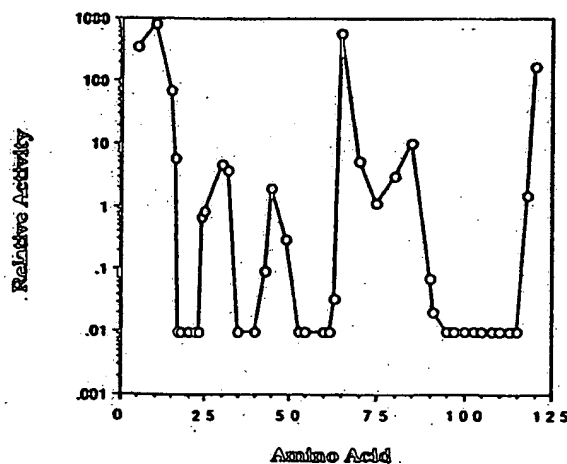
Critical region II. This region is defined by the mutants V7, -8, -29, and -30 and corresponds to amino acids Asn-34 to Ser-41. V7 and -8 are deletions interior to the critical region; V29 and -30 define the maximum N- and C-terminal borders, respectively. V29 has appreciable activity, ≈3.7%, and thus

it is possible that this critical region does not begin until Glu-35 or Glu-36; however, it must contain Glu-36 as this is the most 3' residue contained in V7. Conversely, V30 has very low activity, <0.1%, and thus the C-terminal border is most likely defined by Ser-41.

Critical Region III. This region is defined by deletion mutants VII, -12, -31, -32, -33, and -34 and comprises amino acids Val-52 to Gln-61. Both V11 and -31 are inactive and contain Thr-54, and thus this residue defines the minimum N-terminal boundary of this critical region. The use of additional three-amino acid deletion mutants at this junction is problematical, as they necessarily would include Cys-51 that was shown to be essential for function (see below). The mutants V32, -33, and -34 precisely define the C-terminal border of this critical region as Gln-61. In this overlapping series, V32 is inactive, but V33 and -34 have low activity (≈0.03%).

Critical Region IV. This region is defined by the mutants V19, -20, -21, -22, -23, -36, -37, -38, -39, -40, and -C116 and comprises amino acids Glu-94 to Pro-115. It is the largest critical region in the molecule, spanning 22 amino acids or ≈18% of the mature mGM-CSF sequence. The N-terminal border is defined by V19 and, as in critical region III, is flanked by a critical cysteine residue, Cys-93 (see below). In this case, the amino acids N-terminal to Cys-93 appear to be fairly important for activity, as both V18 and -35 have very low activity (0.07 and 0.02%, respectively). The C-terminal end is defined by either Ile-114 or Pro-115, as both of these residues are contained in V23, and Pro-115 is the final residue in the active VC116 protein product.

Fig. 2 is an illustration of the relative biological activity of all 42 deletion proteins vs. the location of their deletions. This representation reflects the relative contribution of each segment of the molecule to the activity of intact GM-CSF, revealing both critical and noncritical regions. The four critical regions comprise 35% of the residues. The other 65% of the molecule can tolerate small deletions without complete loss of activity. It is important to note that, although 65% of the residues in GM-CSF seem noncritical, this does not imply that these regions can be deleted altogether; as is evident from Fig. 2, even small deletions, such as those generated here, often result in a substantial reduction in activity. Some mutant proteins have an activity that is considerably higher than that of native mGM-CSF. Deletions in these hyperactive



proteins seem to cluster at either terminus. At present we have no explanation for this phenomenon.

The N and C Termini Are Not Important for Activity. *N terminus.* Four N-terminal deletion mutants, V1, -2, -3, and -25, retain biological activity. All except V25, which lies at the border of critical region I, have close to, or greater than, full activity. V25 has appreciable activity (5.6%). These data suggest that the N-terminal 16 amino acids are expendable, and that Glu-17 is noncritical. The mutant VN19, which deletes Pro-2 to Ile-19, was generated to mimic active mutants of human GM-CSF (7). However, we found this mutant protein to be inactive, consistent with our observation that it contained the two N-terminal residues of critical region I.

C terminus. The mutant VC116, which deletes the C-terminal 9 amino acids, is fully active. As the number of deleted amino acid residues decreases, the activity of each successive molecule increases: C118^o increases to 158%; K119^o, encoding Ala-1 to Cys-118, increases to 968%. V24 and -36 have deletions in the expendable C-terminal domain and both yield active molecules, but they have significantly less activity than the corresponding C-terminal "complete" deletion mutants (1.5% for V36 vs. 94% for VC116; 167% for V24 vs. 968% for K119^o).

Sequences Between Critical Regions Are Not Essential for Activity. Segments of GM-CSF that could tolerate small deletions without a total loss of activity were defined as noncritical. Three groups of active mutants can be identified: (i) those of greater or equal activity as wild-type; (ii) those having appreciable, but reduced activity (1–10%); and (iii) those of greatly diminished activity (<1%) (see Fig. 2).

The first group is comprised of almost all N- and C-terminal mutants: V1, -2, and -3 at the N terminus, and V24, K119^o, C118^o, and VC116 at the C terminus. Only mutant V13 at amino acids 64–66, which falls immediately after critical region III, results in a fully active molecule with an internal deletion. The intermediate class includes V5, -6, -9, -14, -15, -16, -17, -25, -28, -29, and -36 (V5 and -28 have close to 1% activity). Although clearly important to the activity of the molecule, the amino acids defined by these deletions are not critical. The last group includes V10, -18, -30, -33, -34, and -35. All fall on the borders of critical regions and have greatly reduced activities. Such mutant proteins are probably of low activity because they may perturb the integrity of the critical regions (see Discussion).

The Disulfide Bridge from Cys-51 to Cys-93 Is Critical for Activity. Protease digestion analysis of h- and mGM-CSF (6) suggests that the four cysteines in GM-CSF pair 1–3 and 2–4. We generated mutant proteins that changed the four individual cysteines to chemically similar serine residues, as well as double mutants that changed both cysteines of the proposed pairs to serines. The activities of each of these mutant proteins are summarized in Table 1.

Changes of either or both Cys-51 and Cys-93 eliminated biological activity. These results show that the formation of this disulfide bond is absolutely required for an active molecule. In contrast, changes of Cys-85 or Cys-118 resulted in active molecules, albeit with lower than wild-type activity. The double mutant C85:118S, in which both Cys-85 and Cys-118 were replaced by Ser, retained full activity. As one or both of these cysteines could be removed without eliminating activity, the disulfide bridge from Cys-85 to Cys-118 is evidently nonessential. The reduction of activity by an unpaired cysteine, as observed for mutant proteins C85S and C118S, in which Cys-85 was replaced by Ser and Cys-118 was replaced by Ser, respectively, may result from formation of inappropriate disulfide linkages.

DISCUSSION

The aim of our work was to delineate regions critical to the activity of mGM-CSF. Four regions of this nature, compris-

ing 35% of the mature protein, were identified using systematic incorporation of deletions into the wild-type sequence. The decision to set the size of the deletions at three amino acids was based on the fine structural deletion map of the lymphokine mouse interleukin 2 (22). In this study, three regions critical to activity were identified by a variety of techniques. A hypothetical deletion analysis of mouse interleukin 2 with various deletion sizes revealed that all three critical regions could be identified using deletions of three amino acids incorporated as infrequently as every 10 amino acids. Larger deletions would require fewer mutants but present the risk of introducing gross structural changes causing inactivation independent of removing functionally important regions. Smaller deletions, although increasing the precision in delineating boundaries, begin to require large numbers of mutants to effectively locate all important regions. The series of deletions used here left not more than two adjacent amino acids unperturbed, making it unlikely that any critical regions was overlooked.

Identification of critical regions exposed by the deletion approach used here requires careful consideration. By deleting any three-residue region, neighboring residues may be forced to occupy the space vacated by the missing residues. This transposition of residues may sometimes result in an inactive mutant as important residues are dislocated from their natural position. This type of secondary effect would result in the identification of these residues as being critical, even though they are not directly responsible for biological function. We cannot rule out this possibility. However, the removed residues are justifiably defined as critical since they are required for an active molecule. With respect to this issue, it is noteworthy that all highly defective mutant proteins (<1% activity) fall on the borders of critical regions. Presumably these proteins have severely reduced activities because they perturb the neighboring critical regions.

To identify amino acid residues that are most important for activity, only those residues deleted in mutant proteins with undetectable activity were scored as critical. A consequence of this arbitrary definition was that deleted residues of some highly defective mutant proteins were considered noncritical. This does not imply that these residues are unimportant: it simply means that these residues are of lesser importance than critical residues.

A synthetic mGM-CSF gene was used as the base template for generating all the mutants used in this study. Although it is not necessary for the methodology employed, we chose to make a synthetic gene because it facilitates examination through other mutational techniques. By identifying a limited number of regions important to an active molecule, it will be possible to focus on these areas using more intensive methods such as saturation mutagenesis and the random replacement of specific amino acids. The presence of unique restriction sites available in a synthetic gene permits convenient access to these regions of interest.

The nature of a critical region, whether it is functionally or structurally important, cannot be distinguished by the deletion method employed here. Extensive analysis of each critical segment will be required to determine their specific roles. However, some information can be obtained by a parallel analysis of the critical regions of m- and hGM-CSF. These proteins are similar molecules, both in primary sequence (~54% identity) and conformation (9). Yet the two polypeptides are species specific. Comparison of the work described here and that of Clark-Lewis *et al.* (7) on synthetic peptide analogs of hGM-CSF shows that the two species share similar domains. The size and relative location of our critical region I coincide with that found in hGM-CSF. Similarities in amino acid composition around this region also imply that these respective critical regions are acting in a like manner. The strongly hydrophobic nature of this portion of

the molecule as well as the observation that Trp-13 (of mouse) is solvent-inaccessible (9) indicate the region is internal. This suggests that critical region I is structurally important. In apparent contradiction with our results, Clark-Lewis *et al.* (7) find that a hGM-CSF molecule extending to residue 95 (the third cysteine) retains residual activity; the corresponding mGM-CSF deletion mutant VC94 has no activity. This discrepancy could reflect a difference in the sensitivity of the assay.

Gough *et al.* (8) have identified residues 11–15, 24–37, 47–49, and 81–89 as required for generating a functional mGM-CSF. The present work and that of Zurawski and Zurawski (22) demonstrate the importance of correlating expression levels of mutant proteins with their corresponding activities. The inability of Gough *et al.* (8) to determine the mutant protein concentrations may explain why none of the critical regions identified in the present work coincide with their results; it is possible that insufficient expression of mutant proteins accounts for their negative assay results. Additionally, their use of large deletions to identify critical regions introduces the potential of gross structural changes that may have resulted in nonspecific inactivation of the mGM-CSF protein.

Circular dichroism measurements (9) and structure predictions using the algorithm of Chou and Fasman (23) suggest that m- and hGM-CSF are composed of both α -helical and β -sheet segments. We have compared the predicted locations of secondary structural components and the critical regions determined by this study. Interestingly, critical regions III and IV are predicted to be β -sheet segments, the borders of which coincide with the boundaries of these critical regions. These regions are each flanked on the N-terminal side by a cysteine residue involved in the formation of the critical disulfide bridge: Cys-51 for critical region III and Cys-93 for critical region IV (Fig. 3). Therefore, this disulfide bond might play a direct role in the arrangement of these critical regions with respect to one another. Since these regions are predicted to be β -sheet segments, it suggests that critical regions III and IV may form a parallel β -sheet structure.

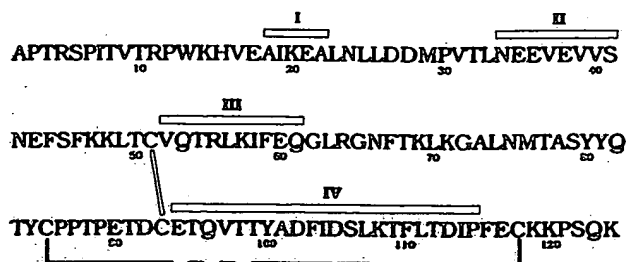


FIG. 3. Salient features of mGM-CSF. The four critical regions are indicated by bars above the primary amino acid sequence. The essential disulfide bridge is shown by the heavy line between the two cysteines that border the N-terminal sides of critical regions III and IV. The other nonessential disulfide bridge is represented by a broken line.

The analysis presented here begins to unravel the different structural and/or functional elements of GM-CSF. Identification of critical regions provides the opportunity to rapidly analyze the essential components of the protein. The knowledge gained from these studies would lead to a better functional understanding of this lymphokine.

We thank Felix Vega for synthesis of DNA, John Abrams for supplying antibodies, and Warren Dang for technical support. We thank Charles Yanofsky for his valuable suggestions on the manuscript. Gerard Zurawski, Ken-ichi Arai, and Phil Hodgkin are thanked for critically reading the manuscript. We also thank Gary Burget for preparation of the manuscript.

1. Metcalf, D. (1986) *Blood* 67, 257–267.
2. Metcalf, D. (1985) *Science* 229, 16–22.
3. Lopez, A. F., Williamson, D. J., Gamble, J. R., Begley, C. J., Harlan, J. M., Klebanoff, S. J., Waltersdorff, A., Wong, J., Clark, S. C. & Vadas, M. A. (1986) *J. Clin. Invest.* 78, 1220–1228.
4. Gough, N. M., Metcalf, D., Gough, J., Grail, D. & Dunn, A. R. (1985) *EMBO J.* 4, 645–653.
5. Lee, F., Yokota, T., Otsuka, T., Gemmel, L., Larson, N., Luh, J., Arai, K. & Rennick, D. (1985) *Proc. Natl. Acad. Sci. USA* 82, 4360–4364.
6. Schrimsher, J. L., Rose, K., Simona, M. G. & Wingfield, P. (1987) *Biochem. J.* 247, 195–199.
7. Clark-Lewis, I., Lopez, A. F., To, L. B., Vadas, M. A., Schrader, J. W., Hood, L. & Kent, S. B. H. (1988) *J. Immunol.* 141, 881–889.
8. Gough, N. M., Grail, D., Gearing, D. P. & Metcalf, D. (1987) *Eur. J. Biochem.* 169, 353–358.
9. Wingfield, P., Graber, P., Moonen, P., Craig, S. & Pain, R. H. (1988) *Eur. J. Biochem.* 173, 65–72.
10. Messing, J. (1983) *Methods Enzymol.* 101, 20–78.
11. Kunkel, T. A., Roberts, J. D. & Zakour, R. A. (1987) *Methods Enzymol.* 154, 367–382.
12. Howard-Flanders, P., Simson, E. & Theriot, L. (1964) *Genetics* 49, 237–246.
13. Ghayeb, J., Kimura, H., Takahara, M., Hsiung, H., Masui, Y. & Inouye, M. (1984) *EMBO J.* 3, 2437–2442.
14. Greenberg, R., Lundell, D., Alroy, Y., Bonitz, S., Condon, R., Fossetta, J., Frommer, B., Gewain, K., Katz, M., Leibowitz, P. J., Narula, S. K., Kastelein, R. & van Kimmenade, A. (1988) *Curr. Microbiol.* 17, 321–332.
15. De Boer, H. A. & Kastelein, R. (1986) in *Biased Codon Usage: An Exploration of Its Role in Optimization of Translation and in Maximizing Gene Expression*, Biotechnology Series, eds. Resnikoff, W. & Gold, L. (Butterworth, Boston), pp. 225–283.
16. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* 74, 5463–5467.
17. Laemmli, U. K. (1970) *Nature (London)* 227, 680–685.
18. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* 76, 4350–4354.
19. Miyajima, A., Orsu, K., Schreurs, J., Bond, M. W., Abrams, J. S. & Arai, K. (1986) *EMBO J.* 5, 1193–1197.
20. Koshland, D. & Botstein, D. (1980) *Cell* 20, 749–760.
21. Mosmann, T. R. (1983) *J. Immunol. Methods* 65, 55–63.
22. Zurawski, S. M. & Zurawski, G. (1988) *EMBO J.* 7, 1061–1069.
23. Chou, P. Y. & Fasman, G. D. (1978) *Annu. Rev. Biochem.* 47, 251–276.

Identification of Residues in the First and Fourth Helices of Human Granulocyte-Macrophage Colony-Stimulating Factor Involved in Biologic Activity and in Binding to the α - and β -Chains of Its Receptor

By Timothy R. Hercus, Bronwyn Cambareri, Mara Dottore, Joanna Woodcock, Christopher J. Bagley, Mathew A. Vadas, M. Frances Shannon, and Angel F. Lopez

Residues within the first and fourth helices of human granulocyte-macrophage colony-stimulating factor (hGM-CSF) were analyzed for their role in biologic activity and interaction with the α - and β -chains of the hGM-CSF receptor. Within the first helix substitution of the surface residues Glu¹⁰, Asn¹⁷, Gln²⁰, Arg²⁰, Arg²⁴, and Asn²⁷ or the buried residues Ala¹⁰, Leu²⁵, and Leu²⁶ did not significantly impair bioactivity or receptor binding. Substitutions at the buried residues Ala²² and Leu²² had intermediate bioactivity. However, substitutions of the surface residue Glu²¹ or the buried residue Ile¹⁹ reduced the relative bioactivity of the analogues to as little as 0.45% and 0.3%, respectively. Substitution of the charged surface residues of the fourth helix showed that

substitution at Glu¹⁰⁰, Lys¹⁰⁷, and Lys¹¹¹ had no significant effect on bioactivity, but substitution at Glu¹⁰² and Asp¹¹² reduced the potency of the analogues to 34% and 7%, respectively. Receptor binding studies showed that, whereas Glu²¹ is the critical residue for binding to the hGM-CSF-receptor β -chain, Asp¹¹² is likely to be involved in binding to the hGM-CSF-receptor α -chain. These results establish the relative contribution of residues in the first and fourth helices for GM-CSF bioactivity and receptor binding, and support a model where the fourth helix of GM-CSF interacts with the α -chain, and the first helix with the β -chain of the GM-CSF receptor.

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HUMAN GRANULOCYTE-macrophage colony-stimulating factor (hGM-CSF) is a pleiotropic cytokine that, by its ability to regulate leukocyte production and function, is likely to play a central role in hemopoiesis, inflammation, and allergy.¹⁻⁴ hGM-CSF exerts its effects by binding to cell surface receptors^{5,6} that have been shown to exist in both a low- and a high-affinity state.⁷ The GM-CSF receptor is composed of at least two chains; a GM-CSF-specific α -chain⁸ that binds with low affinity and a β -chain⁹ common to the receptors for GM-CSF, interleukin-3 (IL-3), and IL-5, which does not bind GM-CSF detectably by itself but confers high-affinity binding when cotransfected with the α -chain. The identification of hGM-CSF residues capable of selectively interacting with the α - or the β -chains of the hGM-CSF receptor and their relevance to function may provide useful clues to dissect hGM-CSF activities and to provide a basis for the construction of hGM-CSF molecules with modified functions.

Determination of the structure of recombinant hGM-CSF (rhGM-CSF) by x-ray crystallography¹⁰ showed that the principal structural feature of hGM-CSF is a four- α -helix bundle with a double overhand topology. This fold is common to many of the known cytokine structures, including macrophage-CSF,¹¹ IL-2,¹² IL-4,¹³ IL-5,¹⁴ and growth hor-

none.¹⁵ This structural conservation among the cytokines is striking considering the low level of sequence similarity.

Previous experiments using a chemical-synthesis approach,¹⁶ neutralizing anti-GM-CSF monoclonal antibodies (MoAbs)¹⁷⁻¹⁹ and human-mouse GM-CSF chimeras^{20,21} have shown that the first and fourth helices of GM-CSF are involved in receptor binding and bioactivity. Recent experiments have shown that the first α -helices of GM-CSF and IL-5 mediate the high-affinity binding of these ligands through interaction with their common receptor β -chain.²² In particular, we have shown that residue Glu²¹ but not Gln²⁰ of hGM-CSF is essential for this interaction.²³ In murine GM-CSF, Lys¹⁴, His¹⁵, and Glu²¹ have been shown to be important for receptor binding and bioactivity.^{24,25}

We have now performed extensive mutagenesis of residues in the first helix of hGM-CSF and substituted the charged surface residues of the fourth helix to determine their relative contribution to receptor binding and bioactivity. Substitution of surface residues within the first helix identified Glu²¹ as the major residue having a significant role in receptor binding and biologic activity. Analysis of buried residues in the first helix suggests that Ile¹⁹ is probably important in maintaining the correct tertiary structure. Substitution of charged surface residues within the fourth helix identified Asp¹¹² as having a role in biologic activity and receptor binding. These data are consistent with a model where Asp¹¹² interacts with the α -chain, and Glu²¹ with the β -chain of the hGM-CSF-receptor complex.

MATERIALS AND METHODS

Site-directed mutagenesis. Site-directed mutagenesis of an hGM-CSF cDNA clone kindly provided by Dr S. Clark (Genetics Institute, Cambridge, MA) was performed in phage M13, as described,²⁶ or by oligonucleotide cassette mutagenesis (OCM). The mutant GM-CSF constructs Q20A^o, E21A, E21R, A22P, R24D, K107D, E108R, K111D, and D112R were generated in phage M13 before being subcloned into the mammalian expression vector pJL4.²⁶ The mutant GM-CSF construct E104A arose as the result of

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* GM-CSF mutants are described using the single-letter amino acid code where the wild-type residue and residue number are listed, followed by the mutant residue.

a serendipitous mutation during a polymerase chain reaction (PCR) amplification. Sequence analysis of the entire GM-CSF PCR product showed the E104A mutation to be a unique substitution.

The remaining mutant GM-CSF constructs were generated using a cassette mutagenesis approach in which short fragments (<60 bp) of wild-type GM-CSF cDNA were replaced by double-stranded oligonucleotide fragments carrying appropriate mutations. Vectors for OCM were prepared by introducing unique restriction sites spanning the first α -helix of the GM-CSF cDNA without altering the encoded protein sequence. Paired combinations of the *Sal* I sites at positions 84 and 126 and an *Xba* I site at position 144 were introduced into the GM-CSF cDNA²⁷ using the phage M13 method. The mutated cDNA was then subcloned into the mammalian expression vector pJL5, a modified form of the pJL4 vector in which the *Bam*HI, *Xba* I, and *Sal* I sites of the polylinker have been destroyed. The resulting pJGMV plasmids express wild-type GM-CSF and were used as vectors for OCM.

Mutagenic oligonucleotides for OCM were synthesized as unique or redundant sequences on an Applied Biosystems 381A DNA Synthesizer (Applied Biosystems, Foster City, CA). Purified, complementary oligonucleotides were 5' phosphorylated and annealed to create double-stranded oligonucleotide fragments that were then ligated to appropriately digested and dephosphorylated pJGMV vectors.

All plasmids carrying GM-CSF mutants were sequenced at the site of mutation before transfection²⁸ and in the OCM-derived mutant constructs, the entire oligonucleotide cassette was sequenced.

Transient expression of GM-CSF and analogues in COS cells. COS cells were cultured and transfected with plasmid constructs as described.²⁹ Briefly, COS cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 20 mmol/L HEPES and glutamine, penicillin, and gentamicin, supplemented with 10% (vol/vol) fetal calf serum (FCS). Cells were harvested when 50% to 70% confluent and were electroporated in a 0.6 mL mixture containing 5×10^5 COS cells, 20 μ g plasmid DNA, 25 μ g sonicated salmon sperm DNA, and 10% FCS, using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, CA) at 300 V and 250 microfarad. After a 24-hour incubation, the medium was replaced with FCS-free DMEM and incubated for a further 72 hours before being harvested and assayed for GM-CSF protein.

Construction of stable Chinese hamster ovary (CHO) cell lines expressing the low-affinity GM-CSF receptor. A cDNA clone for the hGM-CSF-receptor α -chain⁹ was kindly provided by Dr N. Nicola (The Walter and Eliza Hall Institute, Parkville, Victoria, Australia) and cloned into the G-418-selectable, mammalian expression vector pRc/CMV (Invitrogen Corporation, San Diego, CA). CHO cells were grown to approximately 80% confluence in Hams F12 nutrient medium containing penicillin and gentamicin and supplemented with 10% FCS. Linearized plasmid constructs were introduced into CHO cells by electroporation at 1,300 V and 25 μ F using 5×10^5 CHO cells and 10 μ g plasmid DNA. Two days after transfection, selective F12 medium containing 350 μ g/mL G-418 (Geneticin; GIBCO Laboratories, Glen Waverly, Victoria, Australia) was added to the cells. From the G-418-resistant CHO colonies, the A9/C7 cell line stably expressing high levels of the GM-CSF-receptor α -chain was selected and used for low-affinity GM-CSF-receptor binding assays. Scatchard analysis showed from 2 to 5×10^5 binding sites per cell.

Preparation of 125 I-rhGM-CSF. Typically *Escherichia coli*-derived hGM-CSF was radioiodinated by the ICI method²⁹ to a specific activity of approximately 60 μ Ci/ μ g. Iodinated protein was separated from free 125 I by chromatography on a Sephadex G-25 PD-10 column (Pharmacia, Uppsala, Sweden) equilibrated in phosphate-buffered saline (PBS) containing 0.02% (vol/vol) Tween 20 [polyoxyethylene (20)-sorbitan monolaurate]. Before use, the iodinated protein was

purified from Tween by cation-exchange chromatography on a 0.3 mL CM-Sepharose CL-6B column (Pharmacia).

Quantitation and purification of GM-CSF protein. The amount of GM-CSF present in COS cell supernatants was quantified by radioimmunoassay (RIA) using a modification of the procedure described by Lopez et al.²³ Briefly, 125 I-rhGM-CSF (0.1 ng) was mixed with COS cell supernatants and polyclonal rabbit anti-GM-CSF serum and incubated at 4°C for 2 to 4 hours, and an equal volume of reconstituted antirabbit Immunobead reagent (Bio-Rad Laboratories) was added. After a further 2 to 16 hours of incubation at 4°C, the beads were pelleted, washed twice in PBS containing 0.1% (wt/vol) bovine serum albumin (BSA), and counted in a γ -counter (Cobra Auto Gamma; Packard Instrument Co, Meriden, CT). The amount of GM-CSF protein was calculated from a standard curve constructed with known amounts of CHO-derived hGM-CSF (gift from Dr S. Clark, Genetics Institute, Cambridge, MA).

MoAbs to GM-CSF were produced by immunizing BALB/c mice with purified rhGM-CSF. The splenocytes from these mice were fused with Sp2/0 myeloma cells, and the resulting hybridomas³⁰ were screened for the ability to immunoprecipitate 125 I-rhGM-CSF. The positive hybridoma 4D4 was cloned by limiting dilution and used to generate ascites from BALB/c mice. Certain mutants were selected for affinity purification using an immunoaffinity column containing the anti-GM-CSF MoAbs 4D4 or LMM 111,³¹ the latter kindly provided by Dr J. Cebon (Ludwig Institute for Cancer Research, Parkville, Victoria, Australia). Partially purified antibodies were coupled to cyanogen bromide (CNBr)-activated Sepharose 4B beads (Pharmacia) according to the manufacturer's protocol. Purified hGM-CSF analogues were then quantified by RIA.

GM-CSF functional activity. GM-CSF analogues were tested for bioactivity in a proliferation assay. Primary chronic myeloid leukemia (CML) cells from one patient were selected for their ability to incorporate [3 H]thymidine in response to GM-CSF as described.³²

Data analysis. Data from proliferation assays were analysed using the Fig P program (Biosoft, Cambridge, UK). The equation for an asymmetric sigmoid curve that fitted the data was obtained using Fig P, and, from this, the concentration of hGM-CSF analogue required to elicit a biologic response equivalent to the half-maximal response for wild-type hGM-CSF (ED_{50}) was calculated. The biologic activity of the hGM-CSF analogues is expressed as a percentage of wild-type, where wild-type activity is defined as 100%. The percentage of relative GM-CSF activity was calculated using the formula:

$$\frac{\text{Wild-Type hGM-CSF } ED_{50} \text{ (ng/mL)}}{\text{Analogue Dose Equivalent to Wild-Type } ED_{50} \text{ Response (ng/mL)}} \times 100.$$

Using the \log_{10} value of the percentage of relative activity, means and standard deviations were calculated for the individual analogues and expressed as percentage of relative activity.

Radioreceptor assays. Competition for binding to the hGM-CSF high-affinity receptor was determined using freshly purified neutrophils that only express this type of receptor. The cells were suspended in binding medium consisting of RPMI 1640 supplemented with 10 mmol/L HEPES, 0.5% (wt/vol) BSA, and 0.1% sodium azide. Typically, equal volumes (50 μ L) containing 4 to 6×10^6 neutrophils, 100 to 200 pmol/L 125 I-rhGM-CSF, and different concentrations of wild-type GM-CSF or GM-CSF analogues were incubated in siliconized glass tubes for 2 to 3 hours at room temperature.

Competition for binding to the low-affinity GM-CSF receptor was performed using the A9/C7 CHO cell line. A9/C7 cells were detached from flasks by a 5- to 10-minute incubation at room temperature in PBS containing 40 mmol/L EDTA/100 μ g/mL chondroitin sulfate (Calbiochem, La Jolla, CA), were washed, and were resus-

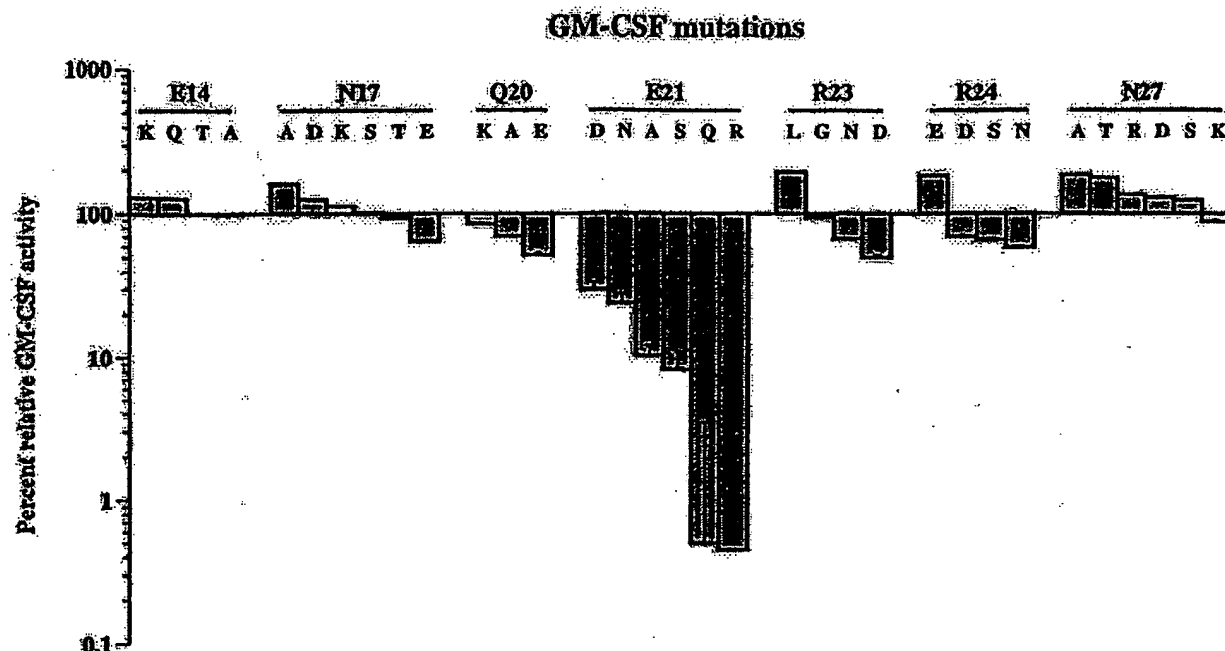


Fig 1. Relative biologic activity of substitution mutants of surface residues within the first helix of hGM-CSF is shown. Bioactivity was assayed using the GM-CSF-dependent proliferation of primary GM cells. Columns represent variation in potency from wild-type hGM-CSF bioactivity for each of the GM-CSF mutants, using the mean from at least two experiments in which full titrations were performed. Potency was defined as the concentration of mutant protein required to elicit a response equivalent to the 50% maximal response induced by wild-type hGM-CSF and is expressed as a percentage of the wild-type hGM-CSF concentration. For certain mutants with low activity, relative potency was determined at a level equivalent to 25% of the maximal response for wild-type hGM-CSF. Bars indicate the single-sided standard deviation from all assays. GM-CSF mutants are described in one letter code, listing the wild-type residue and position above the substitution residues.

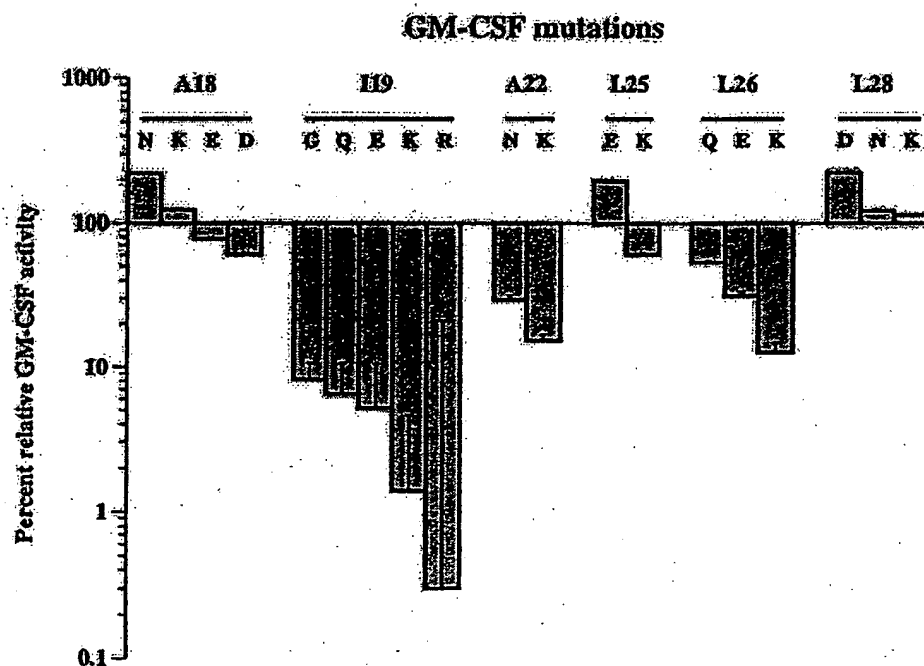


Fig 2. Relative biologic activity of substitution mutants of buried residues within the first helix of hGM-CSF is shown. Bioactivity was determined as described for Fig 1.

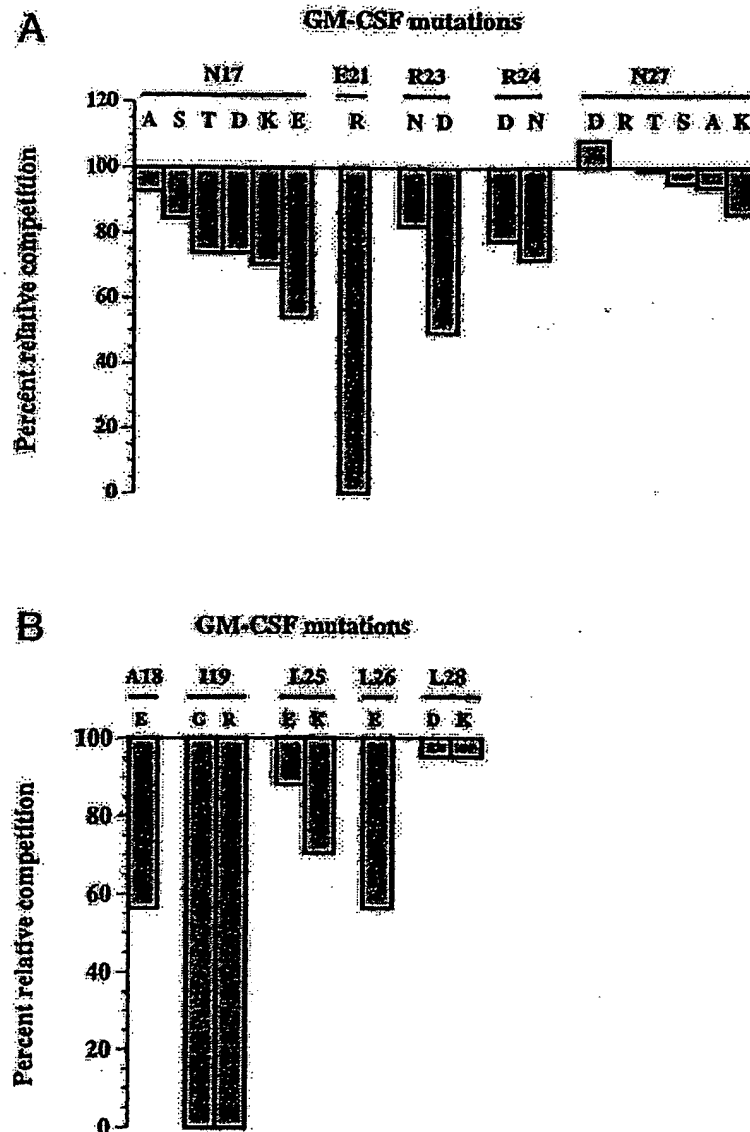


Fig. 3. Competition for ^{125}I -hGM-CSF binding to high-affinity GM-CSF receptors on neutrophils by GM-CSF analogues mutated at (A) surface residues or (B) buried residues. Neutrophils (4×10^5) were incubated at room temperature for 2.5 hours in the presence of ^{125}I -hGM-CSF (150 pmol/L) and a 15-fold excess of wild-type GM-CSF or GM-CSF mutant. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled recombinant hGM-CSF. The percentage competition of each mutant for ^{125}I -hGM-CSF binding was determined from the mean of two replicates and is expressed relative to the competition of wild-type hGM-CSF taken as 100%.

pendent in binding medium. Typically, equal volumes (50 μL) containing 6×10^5 A9/C7 CHO cells, 500 pmol/L ^{125}I -hGM-CSF, and different concentrations of wild-type GM-CSF or GM-CSF analogues were incubated in siliconized glass tubes for 2 to 3 hours at room temperature. In each case, the cell suspensions were overlaid on 200 μL of cold PCS and were centrifuged at room temperature, and the tip of each tube, containing the visible cell pellet, was cut off and counted in a γ -counter. Data from receptor-binding experiments was analyzed using the EBDA/Ligand[®] programs (Biosoft). The percent coefficient of variance (%CV) was determined for wild-type GM-CSF (%CV, 20) and D112R (%CV, 14) binding to human neutrophils.

GM-CSF molecular modelling. The coordinates for GM-CSF were kindly provided by Dr A. Karpus (Cornell University, Ithaca, NY) and were displayed using Insight II software (Biosym Technologies, San Diego, CA).

RESULTS

Biologic activity of mutants in the first helix of hGM-CSF. Residues in the first helix of hGM-CSF were substituted by

different amino acids with a particular emphasis on altering the charge of residues lying on the surface of the helix. Functional analysis of mutations affecting residues on the surface of the first helix indicated that of all exposed residues Glu²¹ was the major residue that contributed significantly to the bioactivity of GM-CSF. The residues Asn¹⁷, Glu²⁰, Arg²³, and Arg²⁴ made only minor contributions as assessed by the GM-CSF-dependent proliferation of primary human CML cells. Interestingly, certain mutations such as N17A, R23L, R24E, and most residue 27 mutants consistently generated higher biologic activity than wild-type GM-CSF. The hierarchy of residue sensitivity and fold decrease in activity (in parentheses) for the most deleterious mutation at each residue was Glu²¹ (220) >> Arg²³ (2) = Glu²⁰ (1.9) > Arg²⁴ (1.7) > Asn¹⁷ (1.5) > Asn²⁷ (1.1) > Glu¹⁴ (1) (see Fig 1).

Analysis of mutations affecting residues of the first helix that are buried in the protein core indicated that these residues are less tolerant to substitution. The hierarchy of residue sensitivity and fold decrease in activity for the most deleteri-

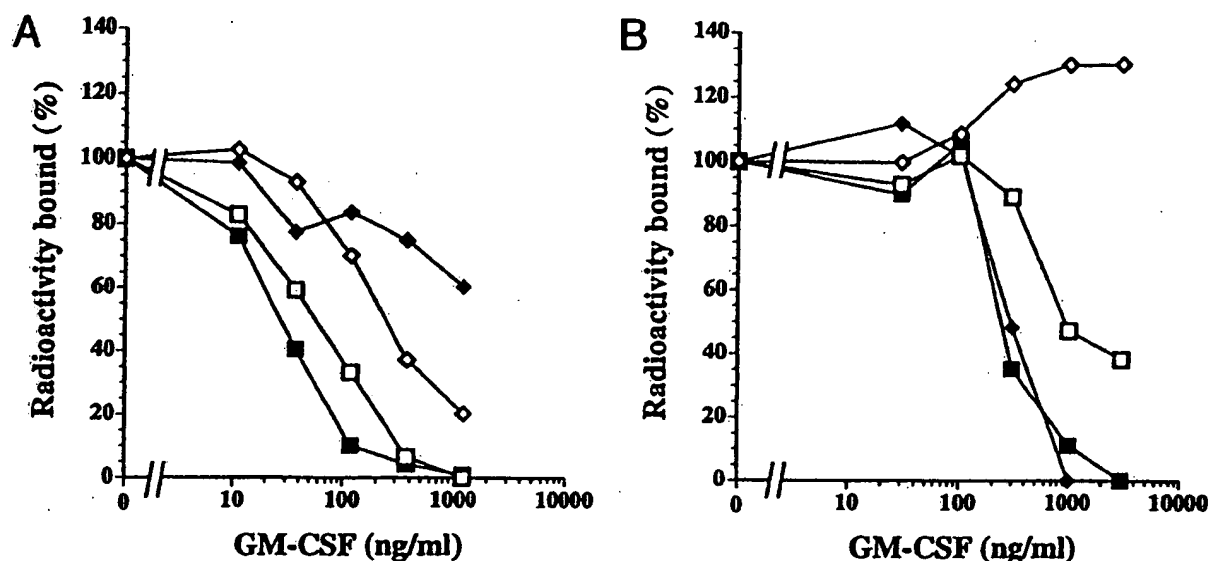


Fig 4. Competition is shown for ^{125}I -rhGM-CSF binding to (A) high-affinity GM-CSF receptors on neutrophils and (B) low-affinity GM-CSF receptors on A9/C7 CHO cells by different concentrations of wild-type GM-CSF (\blacksquare), R23D (\square), E21R (\blacklozenge), and D112R (\circ).

ous mutation at each residue was $\text{Ile}^{19}(330) > \text{Leu}^{26}(7.7) > \text{Ala}^{22}(6.7) > \text{Leu}^{25}(1.6) = \text{Ala}^{18}(1.6) > \text{Leu}^{28}(0.9)$ (see Fig 2). We found that all the GM-CSF analogues mutated at residue 22 expressed very poorly in the COS cell system, yielding approximately 10% of wild-type GM-CSF levels and suggesting that the mutations may have had a deleterious effect on GM-CSF folding or secretion.

Binding of mutants in the first helix of hGM-CSF to the low- (α) and high- ($\alpha + \beta$) affinity GM-CSF receptors. A number of the mutants within the first helix of GM-CSF were tested for their ability to recognize the high-affinity hGM-CSF receptor on human neutrophils. Mutants with a significant reduction in biologic activity also showed a significantly reduced ability to compete for the binding of ^{125}I -rhGM-CSF (Fig 3). Those residues where mutation leads to the most significant reduction in ability to compete for receptor binding were the surface residue Glu²¹ (Fig 3A) and the buried residue Ile¹⁹ (Fig 3B). Representative mutants were selected for more thorough analysis of their ability to compete for the binding of ^{125}I -rhGM-CSF to neutrophils ($\alpha + \beta$ -receptor chains) or A9/C7 CHO cells (α -receptor chain only). From competition curves (shown in Fig 4) for some mutants, we determined the K_d (dissociation constant) of hGM-CSF mutants binding to both high- ($\alpha + \beta$ -chains) and low- (α -chain) affinity hGM-CSF receptors (Table 1). The mutants at positions 17, 23, 24, 27, and 28 bound with essentially wild-type affinity to the high-affinity GM-CSF receptor. These results showed that mutations affecting Ile¹⁹ and Glu²¹ showed the greatest increases in K_d of binding to high-affinity ($\alpha + \beta$) GM-CSF receptors on human neutrophils, corresponding to their decreased biologic activity. In contrast, mutations affecting Ile¹⁹ showed an increased K_d (four-fold) for low-affinity (α only) GM-CSF receptors on A9/C7 CHO cells, whereas mutations of Glu²¹ showed near wild-type binding on A9/C7 cells (Table 1). The effect of Ile¹⁹ substitutions on both α - and $\alpha + \beta$ -chain binding and the

buried nature of this residue suggest that this is a structural residue. Analysis of the change in K_d from α only to $\alpha + \beta$ (ΔK_d), confirms the selective interaction of Glu²¹ with the GM-CSF-receptor β -chain (Table 1).

Mutagenesis of charged residues in the fourth helix of hGM-CSF. Previous experiments have indicated a role for the fourth helix of GM-CSF in bioactivity^{16,20,21} and identification of a critical charged residue on the surface of the first helix suggested that the charged residues on the surface of the fourth helix of hGM-CSF may also play a role in hGM-CSF function. Single amino acid substitutions that either removed or reversed the charge of the residue present in wild-type hGM-CSF were generated. Mutants were assayed

Table 1. Binding Affinities of hGM-CSF First Helix Mutants Tested on Cells Expressing the α - or $\alpha + \beta$ -Chains of the GM-CSF Receptor

Analogue	$\alpha + \beta K_d$ (pmol/L)	α Only K_d (pmol/L)	ΔK_d^*
Wild-type	58	3,700	64
N 17 E	30	ND	—
I 19 G	102	15,700	150
E 21 R	5,000	7,200	1.4
R 23 D	70	5,000	71
R 24 D	41	5,100	124
N 27 D	21	ND	—
L 28 D	39	< ND	—

The K_d values were calculated from a titration curve using increasing concentrations of hGM-CSF analogues to compete for the binding of ^{125}I -rhGM-CSF by using the EBDA/Ligand²³ kinetics program and represent averaged values from multiple experiments. Each concentration of competitor was tested in duplicate using 200 pmol/L ^{125}I -rhGM-CSF mixed with 5×10^6 neutrophils ($\alpha + \beta$) and 500 pmol/L ^{125}I -rhGM-CSF mixed with 6×10^5 A9/C7 CHO cells (α only).

Abbreviation: ND, not done.

* ΔK_d , change in K_d from α -chain only to $\alpha + \beta$.

for bioactivity, and the fold decrease in activity (in parentheses) for each mutant was D112R (14), E108R (3), K107D (1.3), E104A (1.1), and K111D (0.9) (see Fig 5). These results indicated that residue 108 and particularly 112 are functionally important.

The most interesting mutant, D112R was then affinity-purified and tested for its ability to bind the high- and low-affinity hGM-CSF receptors. Competition-binding studies on the high-affinity receptors of human neutrophils (Fig 4A), indicated a K_d of 105 pmol/L for D112R compared with 11 pmol/L for wild-type hGM-CSF, a difference that parallels the reduction in biologic activity. Competition-binding studies on the cloned low-affinity hGM-CSF receptor of A9/C7 CHO cells indicated a K_d of 3.7 nmol/L for wild-type hGM-CSF (Fig 4B and Table 1). However, D112R was unable to compete for 125 I-hGM-CSF binding to A9/C7 CHO cells even when used at a concentration 30-fold in excess of the concentration of wild-type GM-CSF required to achieve 50% competition (Fig 4B). The impaired ability of the Asp¹¹² substitution mutant to bind the low-affinity (α only) hGM-CSF receptor is in marked contrast to Glu²¹ substitutions (Fig 4) and suggests that Asp¹¹² may interact with the GM-CSF-receptor α -chain.

DISCUSSION

There is now considerable evidence to indicate that the amino terminus of GM-CSF is involved in receptor binding and biologic activity.^{16,22} Furthermore, it has been shown that residue Glu²¹ is involved in mediating the high-affinity

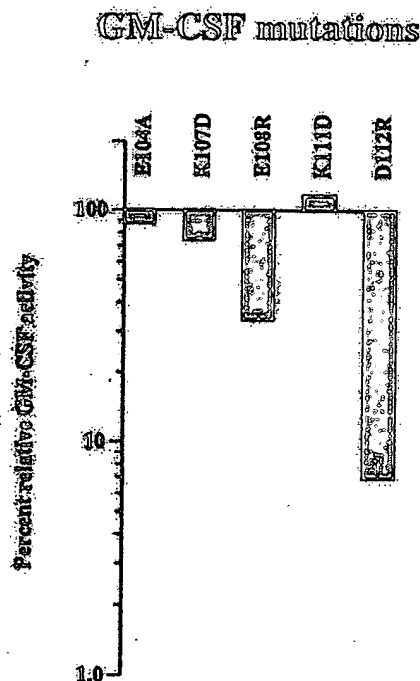


Fig 5. Relative biologic activity of substitution mutants at charged surface residues within the fourth helix of hGM-CSF is shown. Bioactivity was determined as described for Fig 1. GM-CSF mutants are described in one letter code, listing the wild-type residue, number, and substitution.

Table 2. Amino Acid Homology in the Fourth Helix of GM-CSF From Different Species

GM-CSF Species	Fourth Helix	Amino Acid Sequence
Human ²³	103-116	F E S F K E N L K D F L L V
Gibbon ²⁴	103-116	F E S F K E N L K D F L L V
Canine ²⁵	103-116	F K S F K E N L K D F L F R
Bovine ²⁶	102-115	F W N F K E D L K E F L D I
Ovine ²⁷	103-116	F K S F K E N L K D F L F I
Murine ²⁸	103-113	Y A D F I D S L K Y F L T D

The amino acid sequences of the predicted fourth helices are aligned for GM-CSF from six species. All sequences are aligned to the known limits of the GM-CSF fourth helix determined by x-ray crystallography.²⁹ Bored residues are conserved across GM-CSF from all six species.

* Conserved, charged residues.

binding and biologic activity of hGM-CSF.²³ The results presented in this report (Fig 1) establish that of the residues on the surface of the first helix of hGM-CSF, residue 21 is the most important, if not the only, residue interacting with the GM-CSF receptor. So far, this finding seems to be unique to GM-CSF. Human growth hormone contains residues in its first helix that interact with either of the two growth hormone-binding proteins,¹⁵ and similarly, human IL-3 contains residues in the first predicted helix that mediate binding to the receptor α -chain or β -chain.²⁰

Previous studies have also implicated other regions of GM-CSF in function and receptor binding. Mapping the binding epitopes of certain neutralizing MoAbs indicates that residues within the carboxy-terminus (hGM-CSF, 110-127) are involved in GM-CSF-receptor binding,^{18,19} and mutagenesis experiments also emphasized the importance of this region.^{20,21} Data presented in this paper indicate that at least two residues in the carboxy-terminus, Glu¹⁰⁸ and Asp¹¹², are involved in hGM-CSF function and that Asp¹¹² is important for both low- and high-affinity-receptor binding. The observation that charge reversal at residue 112 reduces low-affinity binding (Fig 4) functionally distinguishes this region of GM-CSF from that containing residue 21 and suggests that residue 112 may be involved in the interaction of GM-CSF with the α -chain of its receptor.

It was interesting to note that four of the five charged residues on the surface of the fourth helix show a high degree of conservation among the known GM-CSF species (Table 2), with the only exception being murine GM-CSF where the first and fourth charged residues are not conserved. Our data suggest that the fourth conserved acidic residue in hGM-CSF (Asp¹¹²) may play a role in receptor recognition. This difference between human and murine GM-CSF may be partially responsible for the lack of cross-reactivity observed between the two species of GM-CSF, although the cross-reactivity of bovine GM-CSF in both human and murine bone marrow proliferation assays²⁴ clearly indicates that other residues also play a significant role in determining species specificity.²¹

The carboxy-termini of IL-3²⁰ and IL-5⁴¹ have also been

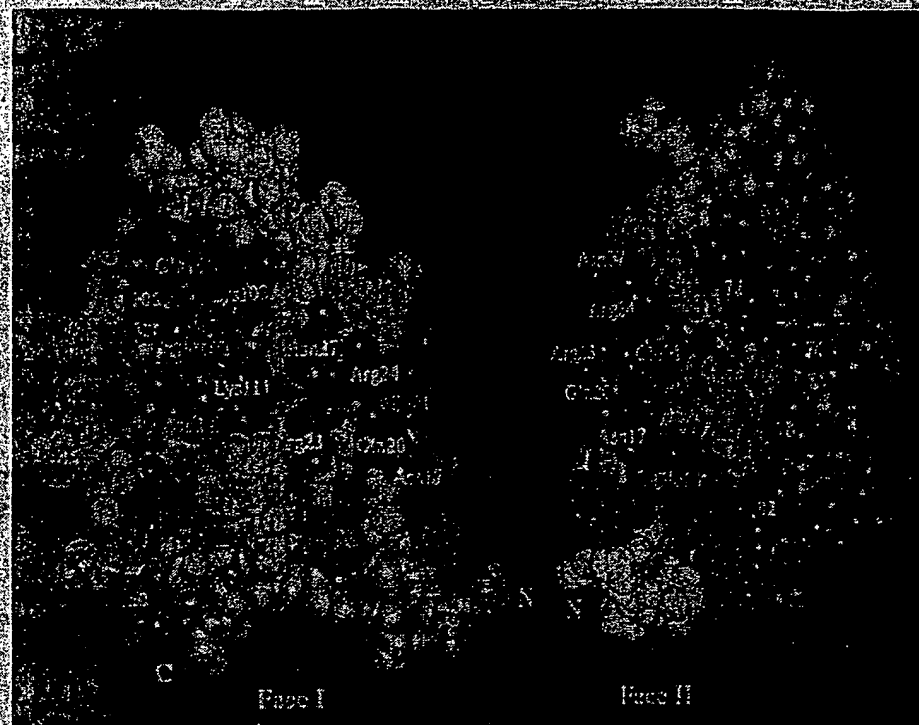


Fig. 8. Two views of the proposed receptor binding sites of human GM-CSF are shown. By analogy with the human growth hormone binding protein interaction face I is the primary receptor binding site (α -chain), whereas face II is the secondary receptor binding site (β -chain). Residues of the proposed receptor binding sites are colored according to the properties of the side chains: Negatively charged (D and E), red; positively charged (K and R), blue; polar (S, T, N, and Q), magenta; nonpolar (C, A, P, G, V, L, I, and M), cyan; and aromatics (H, F, Y, and W), green. Remaining residues are colored white. The images of hGM-CSF from residues 6 to 123 inclusive were generated using Insight II software (Biosym Technologies).

implicated in receptor binding, and an analogue of hIL-3 carrying two carboxy-terminal substitutions has been described that displays a 15-fold enhanced binding to the α -chain of the IL-3 receptor.²⁴ Despite the functional similarity displayed by the carboxy termini of GM-CSF, IL-3, and IL-5, there is no conservation of the residues found on the surface of the fourth helices, consistent with these regions interacting with cytokine-specific receptor α -chains.

A critical assumption in the interpretation of the effects of amino acid substitutions is that the observed differences in biologic activity and receptor binding are caused directly by differences in ligand-receptor contacts and not by conformational changes in the ligand. The observation that low-affinity binding is preserved at wild-type levels in residue 21 mutants that have significantly impaired high-affinity binding and biologic activity suggests that mutations at this position do not alter the overall tertiary structure of hGM-CSF. Indirect evidence that the mutations have not affected the gross tertiary structure of hGM-CSF comes from two observations. The first is that western blot analysis of GM-CSF analogues from the first helix (data not shown) showed no sign of hyperglycosylation that has previously been associated with mutations affecting GM-CSF folding.²⁵ The second is that the polyclonal rabbit anti-GM-CSF serum used for RIA quantitation of the hGM-CSF mutants described in this report is conformation-sensitive and unable to recognize unfolded hGM-CSF.

The data presented here, together with a structural comparison of hGM-CSF and growth hormone,²¹ identify two potential receptor-binding sites on hGM-CSF analogous to the face I/face II sites of growth hormone. Face I of hGM-CSF is proposed to interact with the receptor α -chain and face II

with the receptor β -chain (Fig. 6). In the predicted face I, Asp¹¹² and to a lesser degree Glu¹⁰⁸ may form part of the binding site for the receptor α -chain. Our results show that only one residue, Glu¹¹², on the surface of the first helix of hGM-CSF and lying in the predicted face II receptor binding site is directly implicated in the β -chain interaction. Residues in other helices may also be involved, and inspection of the position of Glu¹¹² in the crystal structure of hGM-CSF (Fig. 6) suggests that residues on the surface of the third helix also lying in the predicted face II receptor binding site might provide contacts between GM-CSF and the β -chain. The mapping of neutralizing MoAb binding epitopes^{12,13} and studies of human-mouse GM-CSF chimeras²⁶ has implicated the region of the third helix of GM-CSF in receptor-binding interactions.

The results presented in this report support a model where face I of GM-CSF and specifically residue 112 interact with the GM-CSF-receptor α -chain. On face II, residue 21 of the first helix interacts with the GM-CSF-receptor β -chain. The functional and binding results shown here provide a structural basis for understanding ligand-receptor interactions and receptor signalling.

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REFERENCES

1. Metcalf D. The granulocyte-macrophage colony-stimulating factors. *Science* 229:16, 1985.

2. Sieff CA, Emerson SG, Donahue RE, Nathan DG, Wang EA, Wong GG, Clark SC: Human recombinant granulocyte-macrophage colony-stimulating factor: A multilineage hematopoietin. *Science* 230:1171, 1985
3. Lopez AF, Williamson DJ, Gamble JR, Begley CG, Harlan JM, Klebanoff SJ, Waltersdorff A, Wong G, Clark SC, Vadas MA: Recombinant human granulocyte-macrophage colony-stimulating factor stimulates in vitro mature human neutrophil and eosinophil function, surface receptor expression, and survival. *J Clin Invest* 78:1220, 1986
4. Haak-Frendscho M, Arai N, Arai K, Baeza ML, Finn A, Kaplan AP: Human recombinant granulocyte-macrophage colony-stimulating factor and interleukin 3 cause basophil histamine release. *J Clin Invest* 82:17, 1988
5. Gasson JC, Kaufman SE, Weisbart RH, Tomonaga M, Golde DW: High-affinity binding of granulocyte-macrophage colony-stimulating factor to normal and leukemic human myeloid cells. *Proc Natl Acad Sci USA* 83:669, 1986
6. Park LS, Friend D, Gillis S, Urdal DL: Characterization of the cell surface receptor for human granulocyte-macrophage colony-stimulating factor. *J Exp Med* 164:251, 1986
7. Elliott MJ, Vadas MA, Eglinton JM, Park LS, To LB, Cleland LG, Clark SC, Lopez AF: Recombinant human interleukin-3 and granulocyte-macrophage colony-stimulating factor show common biological effects and binding characteristics on human monocytes. *Blood* 74:2349, 1989
8. Gearing DP, King JA, Gough NM, Nicola NA: Expression cloning of a receptor for human granulocyte-macrophage colony-stimulating factor. *EMBO J* 8:3667, 1989
9. Hayashida K, Kitamura T, Gorman DM, Arai K, Yokota T, Miyajima A: Molecular cloning of a second subunit of the receptor for human granulocyte-macrophage colony-stimulating factor (GM-CSF): Reconstitution of a high-affinity GM-CSF receptor. *Proc Natl Acad Sci USA* 87:9655, 1990
10. Diederichs K, Boone T, Karplus PA: Novel fold and putative receptor binding site of granulocyte-macrophage colony-stimulating factor. *Science* 254:1779, 1991
11. Pandit J, Bohm A, Jancarik J, Halenbeck R, Kothe K, Kim S: The three-dimensional structure of dimeric human recombinant macrophage colony-stimulating factor. *Science* 258:1358, 1992
12. McKay DB: Unraveling the structure of IL-2. *Science* 257:412, 1992
13. Smith LJ, Redfield C, Boyd J, Lawrence GMP, Edwards RG, Smith RAG, Dobson CM: Human Interleukin 4: The solution structure of a four-helix bundle protein. *J Mol Biol* 224:899, 1992
14. Milburn MV, Hassell AM, Lambert MH, Jordan SR, Proudfoot AEI, Graber P, Wells TNC: A novel dimer configuration revealed by the crystal structure at 2.4 Å resolution of human interleukin-5. *Nature* 363:172, 1993
15. de Vos AM, Uitsch M, Kossiakoff AA: Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. *Science* 255:306, 1992
16. Clark-Lewis I, Lopez AF, To LB, Vadas MA, Schrader JW, Hood LE, Kent SBH: Structure-function studies of human granulocyte-macrophage colony-stimulating factor. *J Immunol* 141:881, 1988
17. Brown CB, Hart CE, Curtis DM, Bailey MC, Kaushansky K: Two neutralizing monoclonal antibodies against human granulocyte-macrophage colony-stimulating factor recognize the receptor binding domain of the molecule. *J Immunol* 144:2184, 1990
18. Nice E, Dempsey P, Layton J, Morstyn G, Cui D, Simpson R, Fabri L, Burgess A: Human granulocyte-macrophage colony-stimulating factor (hGM-CSF): Identification of a binding site for a neutralizing antibody. *Growth Factors* 3:159, 1990
19. Kanakura Y, Cannistra SA, Brown CB, Nakamura M, Seelig GF, Prossie WW, Hawkins JC, Kaushansky K, Griffin JD: Identification of functionally distinct domains of human granulocyte-macrophage colony-stimulating factor using monoclonal antibodies. *Blood* 77:1033, 1991
20. Kaushansky K, Shoemaker SG, Alfaro S, Brown C: Hematopoietic activity of granulocyte-macrophage colony-stimulating factor is dependent upon two distinct regions of the molecule: Functional analysis based upon the activities of interspecies hybrid growth factors. *Proc Natl Acad Sci USA* 86:1213, 1989
21. Shanafelt AB, Johnson KE, Kastelein RA: Identification of critical amino acid residues in human and mouse granulocyte-macrophage colony-stimulating factor and their involvement in species specificity. *J Biol Chem* 266:13804, 1991
22. Shanafelt AB, Miyajima A, Kitamura T, Kastelein RA: The amino-terminal helix of GM-CSF and IL-5 governs high affinity binding to their receptors. *EMBO J* 10:4105, 1991
23. Lopez AF, Shannon MF, Hercus T, Nicola NA, Cambareri B, Dottore M, Layton MJ, Eglinton L, Vadas MA: Residue 21 of human granulocyte-macrophage colony-stimulating factor is critical for biological activity and for high but not low affinity binding. *EMBO J* 11:909, 1992
24. Meropol NJ, Altmann SW, Shanafelt AB, Kastelein RA, Johnson GD, Prystowsky MB: Requirement of hydrophilic amino-terminal residues for granulocyte-macrophage colony-stimulating factor bioactivity and receptor binding. *J Biol Chem* 267:14266, 1992
25. Shanafelt AB, Kastelein RA: High affinity ligand binding is not essential for granulocyte-macrophage colony-stimulating factor receptor activation. *J Biol Chem* 267:25466, 1992
26. Gough NM, Metcalf D, Gough J, Grail D, Dunn AR: Structure and expression of the mRNA for murine granulocyte-macrophage colony-stimulating factor. *EMBO J* 4:645, 1985
27. Wong GG, Wittek JS, Temple PA, Wilkens KM, Leary AC, Luxenberg DP, Jones SS, Brown EL, Kay RM, Orr EC, Shoemaker C, Golde DW, Kaufman RJ, Hewick RM, Wang EA, Clark SC: Human GM-CSF: Molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. *Science* 228:810, 1985
28. Chen EY, Seeburg PH: Supercoil sequencing: A fast and simple method for sequencing plasmid DNA. *DNA* 4:165, 1985
29. Contreras MA, Bale WF, Spar IL: Iodine monochloride (ICl) iodination techniques. *Methods Enzymol* 92:277, 1983
30. Fuller SA, Takahashi M, Hurrell JGR: Preparation of monoclonal antibodies, in Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds): *Current Protocols in Molecular Biology*. New York, NY, Greene and Wiley, 1993, p 11.7.1
31. Cebon J, Dempsey P, Fox R, Kannourakis G, Bonnam E, Burgess AW, Morstyn G: Pharmacokinetics of human granulocyte-macrophage colony-stimulating factor using a sensitive immunoassay. *Blood* 72:1340, 1988
32. Lopez AF, Dyson PG, To LB, Elliott MJ, Milton SE, Russell JA, Juttner CA, Yang Y, Clark SC, Vadas MA: Recombinant human interleukin-3 stimulation of hematopoiesis in humans: Loss of responsiveness with differentiation in the neutrophilic myeloid series. *Blood* 72:1797, 1988
33. Munson PJ, Robard D: LIGAND: A versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* 107:220, 1980
34. Wong GG, Wittek JS, Temple PA, Wilkens KM, Leary AC, Luxenberg DP, Jones SS, Brown EL, Kay RM, Orr EC, Shoemaker C, Golde DW, Kaufman RJ, Hewick RM, Clark SC, Wang EA: Molecular cloning of human and gibbon T-cell-derived GM-CSF cDNAs and purification of the natural and recombinant human proteins. *Cancer cells 3/Growth factors and transformation*. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory, 1985, p 235
35. Nash RA, Schuening F, Appelbaum F, Hammond WP, Boone

- T, Morris CF, Slichter SJ, Storb R: Molecular cloning and in vivo evaluation of canine granulocyte-macrophage colony-stimulating factor. *Blood* 78:930, 1991
36. Maliszewski CR, Schoenborn MA, Cerretti DP, Wignall JM, Picha KS, Cosman D, Tushinski RJ, Gillis S, Baker PE: Bovine GM-CSF: Molecular cloning and biological activity of the recombinant protein. *Mol Immunol* 25:843, 1988
37. O'Brien PM, Rothel JS, Seow H, Wood PR: Cloning and sequencing of the cDNA for ovine granulocyte-macrophage colony-stimulating factor (GM-CSF). *Immunol Cell Biol* 69:51, 1991
38. Gough NM, Gough J, Metcalf D, Kelso A, Grail D, Nicola NA, Burgess AW, Dunn AR: Molecular cloning of cDNA encoding a murine haematopoietic growth regulator, granulocyte-macrophage colony stimulating factor. *Nature* 309:763, 1984
39. Barry SC, Bagley CJ, Phillips J, Dottore M, Cambareri B, Moretti P, D'Andrea R, Goodall G, Shannon MF, Vadas MA, Lopez AF: Two contiguous residues in human interleukin-3, Asp²¹ and Glu²², selectively interact with the α - and β -chains of its receptor and participate in function. *J Biol Chem* (in press)
40. Lokker NA, Zenke G, Strittmatter U, Fagg B, Movva NR: Structure-activity relationship of human interleukin-3: Role of the C-terminal region for biological activity. *EMBO J* 10:2125, 1991
41. McKenzie ANJ, Barry SC, Strath M, Sanderson CJ: Structure-function analysis of interleukin-5 utilizing mouse/human chimeric molecules. *EMBO J* 10:1193, 1991
42. Lopez AF, Shannon MF, Barry S, Phillips JA, Cambareri B, Dottore M, Simmons P, Vadas MA: A human interleukin 3 analog with increased biological and binding activities. *Proc Natl Acad Sci USA* 89:11842, 1992
43. Goodall GJ, Bagley CJ, Vadas MA, Lopez AF: A model for the interaction of the GM-CSF, IL-3 and IL-5 receptors with their ligands. *Growth Factors* 8:87, 1993
44. LaBranche CC, Clark SC, Johnson GD, Ornstein D, Sabath DE, Tushinski R, Pactkau V, Prystowsky MB: Deletion of carboxy-terminal residues of murine granulocyte-macrophage colony-stimulating factor results in a loss of biologic activity and altered glycosylation. *Arch Biochem Biophys* 276:153, 1990
45. Altmann SW, Johnson GD, Prystowsky MB: Single proline substitutions in predicted α -helices of murine granulocyte-macrophage colony-stimulating factor result in a loss in bioactivity and altered glycosylation. *J Biol Chem* 266:5333, 1991

Rational Design of a Mouse Granulocyte Macrophage-Colony-stimulating Factor Receptor Antagonist*

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Mouse granulocyte-macrophage colony-stimulating factor (mGM-CSF) proteins with substitutions at residues in the first α -helix were examined for biological activity and receptor binding properties. Substitution at the buried residue His¹⁶ affected both bioactivity and receptor binding. Of the four surface-exposed positions examined (Arg¹¹, Lys¹⁴, Lys²⁰, and Glu²¹) only substitutions at Glu²¹ impaired bioactivity. Proteins with charge reversal substitutions at this position were partial agonists and weak antagonists of native mGM-CSF action. All substitutions at Glu²¹ abrogated high affinity binding. Lys¹⁴ and Lys²⁰ substitution proteins showed various receptor binding defects. Qualitative and quantitative measurement of these binding defects identified Lys¹⁴ as a residue that interacts specifically with the β subunit of the mGM-CSF receptor, whereas Lys²⁰ appeared to exist at the GM-R α -subunit/GM-R β -subunit interface as substitutions at this position produce both high and low affinity binding losses. These determinations permitted the design of a more potent mGM-CSF antagonist.

Granulocyte-macrophage colony-stimulating factor (GM-CSF)¹ is a pleiotropic cytokine made by various cell types, such as T cells and monocytes, and is likely to play an important role in the regulation of hematopoiesis (1, 2). GM-CSF interacts with specific high affinity cell surface receptors (GM-R $\alpha\beta$, $K_d \sim 5 \times 10^{-11}$ M) that are complexes containing at least two subunits, both of which are members of the cytokine receptor superfamily (3). The receptor α chain (GM-R α) binds GM-CSF specifically with low affinity ($K_d \sim 3 \times 10^{-9}$ M), whereas the receptor β chain (GM-R β), common to the receptors for GM-CSF, IL-3, and IL-5, does not bind this protein detectably by itself, but confers high affinity binding when co-expressed with GM-R α (4-6). Formation of this complex with GM-CSF is required for receptor activation and cellular signaling (7).

Structurally, GM-CSF has been characterized as a four antiparallel α -helical bundle protein (8). This topological fold is shared by many of the known cytokine structures, including interleukins-2 (9), -4 (10), and -5 (11), macrophage colony-

stimulating factor (12), and growth hormone (13).

Molecular genetic studies and studies with neutralizing anti-GM-CSF antibodies have identified regions and residues in both mouse and human GM-CSF that interact with specific subunits of GM-R $\alpha\beta$ (14-21). These studies suggest the functional importance of the first and fourth helix of GM-CSF; in particular, the N-terminal helix of mGM-CSF interacts directly with mGM-R β in the context of mGM-R $\alpha\beta$ to form the high affinity ligand-receptor complex (15). Alanine scanning mutagenesis of this region identified Glu²¹ as essential for this interaction in both mouse and human GM-CSF (16-18). Although substitution of Glu²¹ with alanine in mGM-CSF results in a loss of high affinity binding, biological activity remains essentially intact. The biological activity of hGM-CSF Glu²¹ substitution proteins is reduced, and a basic side chain at this position causes the most severe defect (18).

In this report we examine further the biological and receptor binding properties of mGM-CSF mutant proteins with substitutions at residues in the first helix of mGM-CSF. We show that all Glu²¹ substitution proteins display only low affinity binding, but that the biological activity depends on the nature of the substituted side chain. Substitutions at several other examined residues lead to a loss in either high or low affinity binding. We also show that this knowledge can be used to design mGM-CSF derivatives with antagonistic properties.

MATERIALS AND METHODS

Bacterial Host Strains, Cell Lines, and Vectors—The *Escherichia coli* K12 strains XL-1 (Stratagene) and JM101 (22) were used as host strains for the propagation and maintenance of plasmid and M13 DNA. Strain CJ236 (23) was used to prepare uracil-DNA for use in site-directed mutagenesis procedures. M13mp19 containing the ompA leader sequence and the entire mGM-CSF gene was used as the template for site-directed mutagenesis (24). Strain AB1899 (25) was used as the host for expression of mutant GM-CSF proteins. The plasmid pNIIIompA2 (26) was used as the expression vector for mutant GM-CSF proteins. The use of this secretory *E. coli* system to express biologically active, mature GM-CSF has been described elsewhere (27). A mouse GM-CSF-dependent myeloid leukemia cell line, NFS60, was maintained in RPMI 1640 medium supplemented with 5% (v/v) fetal bovine serum and 50 μ M 2-mercaptoethanol in the presence of mGM-CSF (1 ng/ml) (Schering-Plough).

Construction of mGM-R α Expression Plasmid and Transfection to Mouse L-cells—The mouse GM-R α clone 71 cDNA (6) (kindly provided by L. Park, Immunex) was inserted downstream of the SR α promoter in the expression vector pME18-Neo, a neomycin-resistant derivative of the vector pCEV4 (28) (kindly provided by A. Miyajima, DNAX). Mouse L-cells washed with 20 mM HEPES-buffered saline (pH 7.1) were resuspended at 10^7 cells/ml, and 50 μ g of linearized plasmid DNA was added to 0.8 ml of cell suspension in a 0.4-cm electroporation cuvette (Bio-Rad). Electroporation was performed using a Gene Pulser (Bio-Rad) at 960 microfarads and 400 V. Transfectants were selected with G418 (1.5 mg/ml) (Schering-Plough) and cells expressing mGM-R α were confirmed by their ability to bind ¹²⁵I-mGM-CSF.

Mutagenesis, Recombinant DNA, and Sequencing Protocols—Site-directed mutagenesis was performed using the method described by Kunkel *et al.* (23). Oligonucleotides, 21 nucleotides in length, corre-

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¹ The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; mGM-CSF, mouse GM-CSF; hGM-CSF, human GM-CSF; GM-R, GM-CSF receptor; GM-R α , GM-R α -subunit; GM-R β , GM-R β -subunit; IL-2, interleukin-2; IL-3, interleukin-3; IL-4, interleukin-4; GH, growth hormone; GH-R, GH receptor; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

TABLE I
Characteristics of Lys¹⁴ substitution proteins

Sample ^a	EC ₅₀ ^b	K _{dhigh} ^c	(%CV) ^d	K _{dlow} ^e	(%CV) ^d	One site vs. two site ^f		n ^g	% activity ^h	n ^h	% binding ⁱ	
						F	p				High	Low
GMCSF	5.7E-13	2.5E-11	9	9.9E-09	25	119.67	0	3	100	7	100	100
K14A	3.9E-13	9.2E-11	8	2.1E-07	118	12.15	0.01	3	>30	1	~5-20	~1-5
K14E	3.9E-13	8.2E-10	8	ND ^j		-3.26	1	3	>30	3	~1-5	
K14F	5.2E-13	7.9E-11	4	6.1E-08	15	405.31	0	3	>30	2	~5-20	~5-20
K14G	2.7E-13	3.9E-10	4	ND ^j		-3.28	1	3	>30	3	~5-20	
K14H	4.4E-13	4.4E-11	10	1.3E-08	36	62.09	0	3	>30	2	>30	>30
K14L	3.9E-13	5.4E-11	13	1.6E-07	109	13.3	0	3	>30	3	>30	~1-5
K14M	3.8E-13	2.5E-11	10	7.7E-07	31	81.97	0	3	>30	3	>30	>30
K14P	3.7E-13	2.3E-09	16	ND ^j		2.11	0.19	3	>30	2	~1-5	
K14Q	3.2E-13	7.9E-11	22	2.7E-08	86	11.4	0.01	3	>30	3	~10-30	~10-30
K14R	4.5E-13	5.3E-11	11	7.0E-09	23	111.96	0	3	>30	3	>30	>30
K14S	2.1E-13	6.0E-10	15	ND ^j		-3.39	1	3	>30	2	~5-20	
K14V	4.9E-13	2.9E-10	26	ND ^j		1.96	0.19	3	>30	2	~10-30	
K14W	3.5E-13	5.5E-10	9	ND ^j		1.41	0.31	3	>30	3	~5-20	
K14Y	7.0E-13	2.8E-10	13	ND ^j		-1.81	1	3	>30	2	~10-30	

^a Mutant proteins are identified by their native single-letter amino acid code, residue number, and amino acid substitution.

^b The concentration of each protein that gave 50% maximal response was determined using the Softmax program (Molecular Devices) from the data presented in Fig. 1, panel 2A.

^c High and low refer to the high affinity and low affinity equilibrium binding constants calculated using the Ligand program (33) from the data presented in Fig. 1, panel 2B.

^d The percentage coefficient of variations is the standard error given by the Ligand program (33) for the K_d value presented.

^e F and p values determined by the Ligand program (33) indicate that substitutions at Lys¹⁴ can result in either one or two binding site model.

^f Number of NFS60 proliferation assays performed.

^g Percent activities of the mutant proteins relative to the EC₅₀ of wild-type mGM-CSF.

^h Number of NFS60 competition binding assays performed.

ⁱ Percent high and low affinity binding relative to values determined for wild-type mGM-CSF.

^j Not determined. Error messages from the Ligand program (33) indicated that the two-site model was unacceptable for the data.

sponding to mGM-CSF sequences incorporating the desired amino acid substitution were used as primers in the mutagenesis reactions. Individual clones were sequenced using the method and modifications described in the Sequenase (United States Biochemical Corp., Cleveland, OH) protocol (29). M13 (replicative form) DNA containing confirmed mutations was digested with XbaI and BamHI and subcloned into pNIIIompA2.

Preparation and Quantitation of Protein Samples—Expression and quantitation of mutant proteins has been described previously (24). Briefly, mutant proteins were expressed in *E. coli* AB1899 and periplasmic extracts were generated by osmotic shock. The concentration of mutant protein in the periplasmic extracts was determined by quantitative immunoblot analysis. Extracts were titrated along with a standard curve of purified *E. coli* derived recombinant GM-CSF. Either hybridoma mg 1.8.2 (30) or 22E9 (31) were used as primary antibody, and the secondary antibody was ¹²⁵I-labeled sheep anti-rat IgG (Amersham Corp.). The amount of immunoreactive protein was quantified by integrating the area × density from an exposed screen using a PhosphorImager (Molecular Dynamics). The error in the calculated concentrations of GM-CSF mutant proteins by this method did not exceed 2-fold based on repetitive analysis of individual samples.

Biological Assays for GM-CSF Activity—Protein extracts were assayed for their ability to stimulate the proliferation of the mouse GM-CSF-dependent cell line NFS60. Samples were titrated in quadruplicate using serial 3-fold dilutions. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) assay (32) was used to measure the extent of proliferation, and the absorbance value difference of 570–650 nm was measured with a V_{max} kinetic microplate reader (Molecular Devices). The concentration of each mutant and wild-type mGM-CSF protein that gave 50% maximal response was determined using the Softmax program (Molecular Devices). The correlation coefficient of the 4-parameter curve fit to the measured values exceeded 0.990 for all curves presented. Protein extracts were assayed for their ability to antagonize the GM-CSF proliferative response of NFS60 cells. Samples were titrated in quadruplicate using serial 3-fold dilutions in the presence of 3.5 × 10⁻¹² M mGM-CSF using the MTT assay format.

Competitive Receptor Binding Assays of Mutant GM-CSF Proteins—Mouse GM-CSF was labeled with ¹²⁵I using the Bolton and Hunter reagent (Amersham; specific activity ~400 mCi/mmol) to a specific activity of ~20–40 μCi/μg as described previously (16). Competition binding assays were performed as follows: NFS60 cells maintained in media containing mGM-CSF were harvested, washed, and incubated with 1 ml of ice-cold 10 mM NaPO₄, 150 mM NaCl (pH 3.0) for 2 min then

diluted to 50 ml with 10 mM NaPO₄, 150 mM NaCl (pH 7.0). Following centrifugation the cells were resuspended in 1 × Hanks' balanced salts solution (Life Technologies, Inc.) containing 0.1% bovine serum albumin, 0.02% NaN₃, and 10 mM HEPES (pH 7.5) (HBAH buffer). 2.5 × 10⁶ cells in 200 μl of HBAH buffer were incubated with decreasing concentrations of unlabeled competitor in the presence of 3.0 × 10⁻¹¹ M ¹²⁵I-mGM-CSF at 4 °C with continuous agitation for 4 h. Samples were titrated in triplicate using serial 3-fold dilutions. Nonspecific binding was determined from the data collected for the competition of unlabeled wild-type mGM-CSF. Cell-bound radioactivity was separated from free ligand by centrifugation at 4 °C (2 min, 12,000 × g) through diethylphthalate/dibutylphthalate (2:3). Bound and total radioactivity was measured with a Cobra 5010 γ-counter (Packard). Low affinity receptor competition binding assays were performed in a similar manner except 7.5 × 10⁵ mouse L-cells expressing mGM-Rα were incubated with unlabeled competitors in the presence of 2.0 × 10⁻⁹ M ¹²⁵I-mGM-CSF for 2 h. The equilibrium binding data were analyzed using the Ligand program (33). Curve fitting for both one- and two-site models were performed on each sample. Curves were either fit using a fixed high and low affinity equilibrium binding constants (K_{dhigh}: 5.0 × 10⁻¹¹ M, K_{dlow}: 3.0 × 10⁻⁹ M) or by fitting the control wild-type mGM-CSF and the mutant protein competition curve simultaneously. Binding curves are presented as the concentration of competitor versus the specific counts/minute bound as a percentage of total bound. Analysis of the binding data for Lys¹⁴ substitutions exposed a limitation of the Ligand software. Substitutions for Lys¹⁴ showed a range of defects in high affinity binding. For substitutions resulting in small changes a two site model with reliable high and low affinity K_d values was statistically significant. For substitutions with reduced high affinity binding approaching that of the low affinity, the Ligand program was unable to discriminate a two-site model. This is reflected in the unreliable binding constants with small F and large p (>0.05) values as well as high affinity K_d values with values higher than would be expected from examination of the binding curves (Table I). We presume that this is the result of combining both high and low affinity values into a single binding constant. Only by measuring the affinity of Lys¹⁴ substitution proteins for the low affinity receptor alone (mGM-Rβ) were we able to assign binding defects of Lys¹⁴ mutant proteins to the mGM-Rβ (Table IV).

Mouse GM-CSF Molecular Modeling—The coordinates for hGM-CSF were kindly provided by Dr. A. Karplus (Cornell University, Ithaca, NY). The mouse GM-CSF Cα backbone conformation was modeled using SegMod (34) followed by refinement of side chain conformations using the program CARA (35) within the LOOK suite of programs

(Molecular Applications Group, Palo Alto, CA). The structure was displayed using Insight II software (Biosym Technologies, San Diego, CA).

RESULTS

Biological Response of mGM-CSF Mutant Proteins—A panel of amino acid substitutions was generated for selected residues in the first α -helix of mGM-CSF. Based on previous studies (16) the following residues were targeted: Arg¹¹ (R11), Lys¹⁴ (K14), His¹⁵ (H15), Lys²⁰ (K20), and Glu²¹ (E21). Position Glu¹⁷, although surface exposed and located between Glu¹⁴ and Glu²¹, was not included in this analysis, since all available evidence points to at best a minor involvement of this residue (15, 16). Mutant protein was produced in *E. coli* and quantitated as described previously (24). Biological activity was assessed by measuring the proliferative response of the mGM-CSF-dependent myeloid cell line NFS60. Of the four surface-exposed residues (Arg¹¹, Lys¹⁴, Lys²⁰, and Glu²¹), only substitution of Glu²¹ significantly altered the biological activity of mGM-CSF (Fig. 1, panel 5A, Table III). Charge reversal substitutions at this position (E21R and E21K) reduced the biological activity to <1% and reduced the magnitude of the plateau. Several other Glu²¹ substitution proteins (Ala, Gly, Ser, and Leu) had lower biological activity (10–30%), although the magnitude of the response was not affected. All other substitutions at these four positions had only a marginal effect on the biological activity (>30%) with the exception of proline substitutions of Lys²⁰ and Glu²¹. The introduction of proline residues at these positions in the first α -helix reduced the activity of the resulting proteins to less than 1% (Fig. 1, panels 4A and 5A, Tables II and III). The magnitude of the plateau for E21P but not K20P was reduced. Proline substitutions of Arg¹¹ and Lys¹⁴, located just outside and just within the first α -helix, respectively, did not significantly alter the biological activity (Fig. 1, panels 1A and 2A, Table I).

Based on homology to hGM-CSF, His¹⁵ of mGM-CSF appears to occupy a buried position in the first α -helix. This buried position is less tolerant to substitution than the surface exposed residues (with the exception of Glu²¹). Substitution at His¹⁵ with charged or polar residues resulted in a reduced biological activity, whereas hydrophobic or aromatic substitutions are well tolerated (Fig. 1, panel 3A). All His¹⁵ mutants reached full plateau.

Competitive Receptor Binding of mGM-CSF Mutant Proteins—The effects of amino acid substitution on the ability of mGM-CSF to bind to the high affinity mGM-CSF receptor (mGM-R β) on NFS60 cells was measured using conditions designed to detect both high and low affinity binding (see "Materials and Methods"). Some proteins had no loss in high affinity binding, and such mutant proteins were not investigated further. Some proteins had decreased high affinity binding, and the mutant protein was then tested for its ability to bind to the low affinity mGM-CSF receptor (mGM-R α) on stably transfected L-cells (see "Materials and Methods"). This step was necessary so that the observed reduction in affinity could be assigned to either a loss of binding to the mGM-R β component in the mGM-R $\alpha\beta$ complex, to a loss of binding to the mGM-R α component, or to a loss of binding to both.

For most substitution mutants at Arg¹¹, Lys¹⁴, His¹⁵, and Lys²⁰, a two-site fit of the high affinity receptor binding data was statistically significant (data shown for Lys¹⁴ and Lys²⁰; Tables I and II). Substitution at Arg¹¹ with either a negatively charged (R11E) or positively charged (R11K) residue, neutral (R11G), hydrophobic (R11L), or aromatic (R11W) residue had no effect on either high or low affinity binding constants (data not shown). This residue was not studied further.

Mutant proteins with charged and polar substitutions at position His¹⁵ showed a reduced ability to compete for ¹²⁵I-

mGM-CSF (Fig. 1, panel 3B), in agreement with the observed loss in biological activity (Fig. 1, panel 3A). Since this is a buried position in the protein, losses in activity and binding are probably due to structural perturbations of the protein core. Proteins mutated at this position were also not studied further.

Mutations at position Lys¹⁴ did not alter noticeably the biological activity of the resulting mutant protein. However, significant binding defects were observed (Fig. 1, panels 2A and 2B, Table I). For some Lys¹⁴ mutants, only one affinity site is detected (Glu, Gly, Ser, Val, Trp, and Tyr). For others, two sites were still detected but both were reduced in affinity (e.g. Phe and Gln). In general, reduction of the high affinity binding constant was less than 10-fold, while values for the low affinity K_d showed wider variation with a concomitant increase in the %CV, indicating that the calculated value for the low affinity binding constant was less reliable under these conditions. Several Lys¹⁴ mutants displaying a range of high affinity defects were selected and analyzed for their low affinity binding to mGM-R α expressed alone on stably transfected L-cells (Fig. 2A). Of the five mutant proteins tested, four had a low affinity K_d similar to mGM-CSF ($K_d = 7.2 \times 10^{-9}$ M; Table IV). From this analysis we conclude that the reduction seen in the high affinity binding constant for Lys¹⁴ substitution proteins results from losses in binding to the mGM-R β in the mGM-R $\alpha\beta$ complex. Protein K14P is the exception. A proline substitution at this position affected the low as well as the high affinity binding constant (Table I).

Mutant proteins with Lys²⁰ substitutions also showed losses in both high and low affinity binding as measured on NFS60 cells (Fig. 1, panel 4B, Fig. 2B, Tables II and IV). To distinguish between losses in binding to the mGM-R β and/or mGM-R α , Lys²⁰ substitution proteins were analyzed on mGM-R α -expressing L-cells. Whereas most substitutions at Lys²⁰ did not affect the low affinity binding constant as measured on L-cells, small amino acid substitutions (Ala, Gly, and Ser) at this position led to a 10-fold reduction in mGM-R α binding (Fig. 2B, Table IV). The introduction of proline at this position profoundly disturbed the binding of the resulting protein to both high and low affinity receptor.

In contrast to the other mutant proteins examined, all Glu²¹ substitution proteins competed ¹²⁵I-mGM-CSF for only a single class of binding sites on NFS60 cells (Fig. 1, panel 5B, Table III). The affinity of Glu²¹ substitution proteins ($K_d = 1-4 \times 10^{-9}$ M) for these sites suggested that binding is to mGM-R α . Scatchard analysis of one of the Glu²¹ substitution proteins, E21A, has shown that both affinity and number of binding sites were consistent with binding of this mutant protein to mGM-R α only (16). Again, the exception was E21P. This protein had a low affinity binding constant that was at least three orders of magnitude higher than the other Glu²¹ proteins (Fig. 5B, Table III), probably caused by severe structural changes (36).

Design of mGM-CSF Antagonists—Mutant proteins E21R and E21K were unable to trigger a maximal biological response. Such partial agonists should be antagonists of mGM-CSF at concentrations equal to or greater than those at which they bind the receptor. E21K was a partial agonist with about 40% residual activation on NFS60 cells (Fig. 3). At concentrations which elicited the partial agonist activity, E21K was an antagonist of mGM-CSF activity (Fig. 4). However, its efficacy was limited by its relatively high residual activation level. We reasoned that the residual ability of E21K to activate mGM-R $\alpha\beta$ might result from contacts with mGM-R β which were still intact in E21K. Identification of Lys¹⁴ as a residue that exclusively interacts with mGM-R β suggested the possibility that a further reduction of the submaximal response of E21K could be achieved by combining mutations at these two positions. Fig. 3

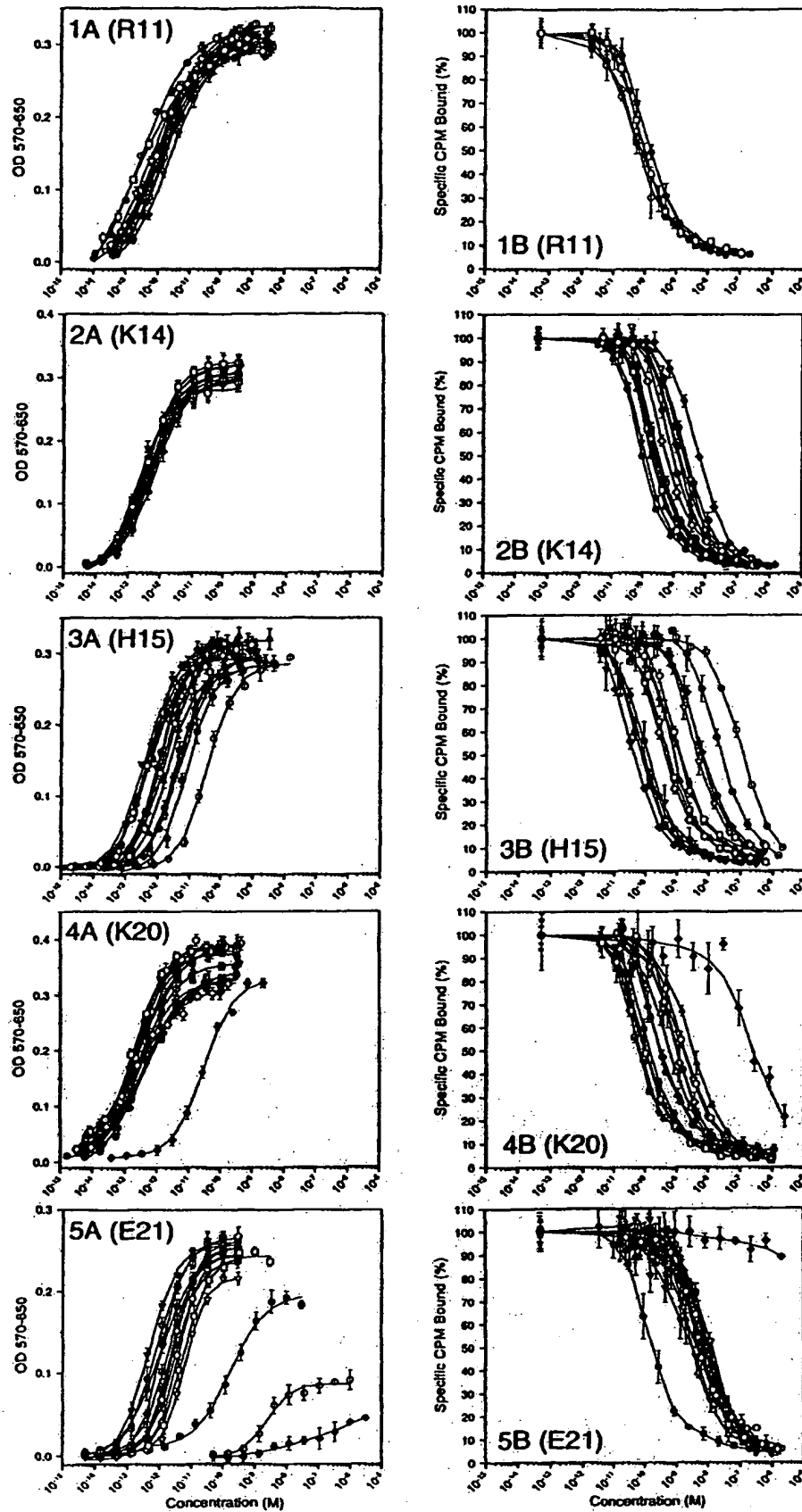


FIG. 1

TABLE II
Characteristics of Lys²⁰ substitution proteins

Sample ^a	EC ₅₀ ^b	K _{dhigh} ^c	(%CV) ^d	K _{dlow} ^c	(%CV) ^d	One site vs. two sites ^e		n ^f	% activity ^g	n ^h	% binding ⁱ	
						F	p				High	Low
GMCSF	2.3E-13	3.8E-11	10	5.9E-08	68	22.1	0	2	100	9	100	100
K20A	3.4E-13	4.6E-10	11	1.1E-07	47	36.5	0	2	>30	4	~5-20	~10-30
K20E	2.2E-13	2.3E-10	11	3.1E-07	131	8.6	0.01	2	>30	3	~5-20	>30
K20F	2.6E-13	1.3E-11	19	6.8E-08	23	123.1	0	2	>30	2	>30	>30
K20G	5.0E-13	1.1E-09	16	1.2E-06	376	5	0.45	2	>30	3	>30	~5-20
K20H	3.2E-13	1.9E-11	7	1.3E-08	28	102.3	0	2	>30	2	>30	>30
K20L	2.1E-13	1.6E-11	27	2.2E-09	63	15.3	0	2	>30	2	>30	>30
K20M	2.1E-13	2.2E-11	23	1.3E-08	68	16.7	0	2	>30	2	>30	>30
K20P	3.1E-11	ND ^j		9.3E-08	25			2	~0.2-0.8	3		~5-20
K20Q	2.4E-13	2.1E-10	12	2.5E-07	76	24	0	2	>30	3	~5-20	~5-20
K20R	3.9E-13	1.7E-11	18	3.1E-09	54	24.4	0	2	>30	2	>30	>30
K20S	3.8E-13	3.6E-10	23	2.2E-08	59	17.9	0	2	>30	2	~5-20	>30
K20V	1.7E-13	3.3E-11	14	1.2E-08	30	71.9	0	2	>30	3	~10-30	>30
K20W	2.7E-13	1.1E-11	13	5.4E-09	25	113.5	0	2	>30	3	>30	>30
K20Y	4.1E-13	8.3E-11	11	1.1E-07	31	82.8	0	2	>30	3	~10-30	~5-20

^a Mutant proteins are identified by their native single-letter amino acid code, residue number, and amino acid substitution.^b The concentration of each protein that gave 50% maximal response was determined using the Softmax program (Molecular Devices) from the data presented in Fig. 1, panel 4A.^c High and low refer to the high affinity and low affinity equilibrium binding constants calculated using the Ligand program (33) from the data presented in Fig. 1, panel 4B.^d The percentage coefficient of variations is the standard error given by the Ligand program (33) for the K_d value presented.^e F and p values determined by the Ligand program (33) indicate that the fit using a two binding site model was statistically significant except for K20G and K20P.^f Number of NFS60 proliferation assays performed.^g Percent activities of the mutant proteins relative to the EC₅₀ of wild-type mGM-CSF.^h Number of NFS60 competition binding assays performed.ⁱ Percent high and low affinity binding relative to values determined for wild-type mGM-CSF.^j Not determined. Error messages from the Ligand program (33) indicated that the two-site model was unacceptable for the data.TABLE III
Characteristics of Glu²¹ substitution proteins

Sample ^a	EC ₅₀ ^b	K _{dhigh} ^c	(%CV) ^d	K _{dlow} ^c	(%CV) ^d	One site vs. two sites ^e		n ^f	% activity ^g	n ^h	% binding ⁱ	
						F	p				High	Low
GMCSF	1.2E-12	5.90E-11	14	1.2E-09	62	21.5	0	3	100	7	100	100
E21A	4.0E-12	ND ^j		3.5E-09	20			8	~10-30	3		>30
E21F	1.7E-12	ND ^j		4.8E-09	11			3	>30	2		>30
E21G	3.2E-12	ND ^j		3.1E-09	13			3	~10-30	2		>30
E21H	2.2E-12	ND ^j		3.6E-09	10			3	>30	2		>30
E21K	8.0E-10	ND ^j		3.2E-09	7			3	~0.1-0.5	3		>30
E21L	5.7E-12	ND ^j		3.2E-09	13			3	~10-30	2		>30
E21M	2.6E-12	ND ^j		2.7E-09	7			3	>30	1		>30
E21P	1.0E-06	ND ^j		<1E-06	251			3	<.01	1		<.01
E21Q	8.2E-13	ND ^j		1.4E-09	17			3	>30	2		>30
E21R	1.8E-10	ND ^j		4.4E-09	9			3	~0.3-1.0	2		>30
E21S	6.5E-12	ND ^j		4.4E-09	4			3	~10-30	1		>30
E21V	3.0E-12	ND ^j		4.0E-09	21			3	>30	1		>30
E21W	4.8E-13	ND ^j		2.8E-09	9			3	>30	3		>30
E21Y	1.2E-12	ND ^j		2.6E-09	25			3	>30	2		>30

^a Mutant proteins are identified by their native single-letter amino acid code, residue number, and amino acid substitution.^b The concentration of each protein that gave 50% maximal response was determined using the Softmax program (Molecular Devices) from the data presented in Fig. 1, panel 5A.^c High and low refer to the high affinity and low affinity equilibrium binding constants calculated using the Ligand program (33) from the data presented in Fig. 1, panel 5B.^d The percentage coefficient of variations is the standard error given by the Ligand program (33) for the K_d value presented.^e F and p values could not be determined. The Ligand program (33) could only fit the data to a single, low affinity binding site.^f Number of NFS60 proliferation assays performed.^g Percent activities of the mutant proteins relative to the EC₅₀ of wild-type mGM-CSF.^h Number of NFS60 competition binding assays performed.ⁱ Percent high and low affinity binding relative to values determined for wild-type mGM-CSF.^j Not determined. Error messages from the Ligand program (33) indicated that the two-site model was unacceptable for the data.

FIG. 1. Biological activity and competition binding of wild-type and mutant proteins. A, proliferation of NFS60 cells was measured as a function of protein concentration using the MTT assay (32). Assays were performed in quadruplicate with symbol error bars indicating standard deviation. Curves were generated using a 4-parameter logistical fit and all correlation coefficients exceeded 0.990. B, competition of ¹²⁵I-mGM-CSF on NFS60 cells were performed with a constant concentration of 3.0×10^{-11} M ¹²⁵I-mGM-CSF in triplicate with symbol error bars indicating standard deviation. Under these conditions both high and low affinity sites were detected using the Ligand program (33). Each pair of A and B panels represents multiple amino acid substitutions for a specific residue as indicated Arg¹¹ (1), Lys¹⁴ (2), His¹⁶ (3), Lys²⁰ (4), and Glu²¹ (5). Substitutions are represented by each symbol as follows: ●, wild-type; ▲, Gly; ▼, Met; ◆, Pro; ○, Ala; □, Leu; ◇, Val; △, His; ▤, Phe; ▥, Trp; ◇, Tyr; Δ, Gln; ▽, Ser; ○, Arg; ○, Glu; ○, Lys.

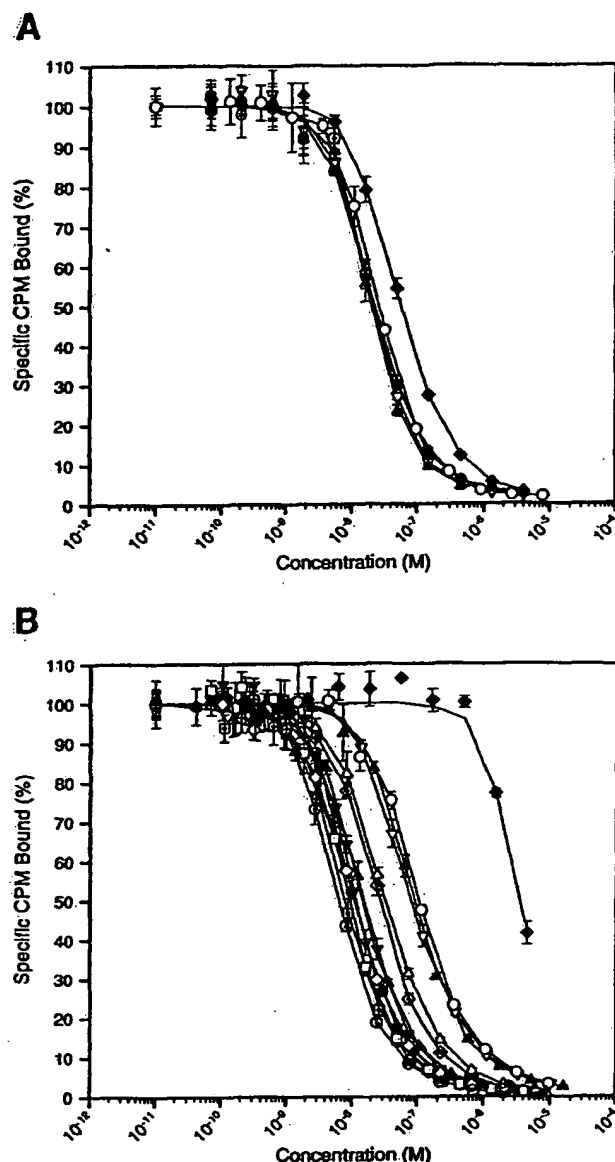


Fig. 2. Competition binding of mutant and wild-type mGM-CSF proteins. A, competition of ^{125}I -mGM-CSF on L cells transfected with mGM-R α by \bullet , mGM-CSF; \circ , K14A; \oplus , K14E; Δ , K14G; \diamond , K14P; and ∇ , K14S. B, competition of ^{125}I -mGM-CSF on L cells transfected with mGM-R- α by \bullet , mGM-CSF; Δ , K20G; ∇ , K20M; \diamond , K20P; \circ , K20A; \square , K20L; \circ , K20V; \boxplus , K20F; Δ , K20H; ∇ , K20W; \oplus , K20Y; Δ , K20Q; ∇ , K20S; \oplus , K20R; \oplus , K20E. Assays were performed in triplicate using a constant concentration of 2.0×10^{-9} M ^{125}I -mGM-CSF with symbol error bars indicating standard deviation.

shows that the K14E, E21K double mutant protein had, in fact, lost almost (>90%) all ability to activate mGM-R α on NFS60 cells. As expected, this protein showed much improved efficacy when used on NFS60 cells to antagonize mGM-CSF (Fig. 4).

DISCUSSION

The involvement of the amino-terminal region of mGM-CSF in receptor binding and biological activity is well documented (16, 17). In particular, it is now clear that residues in the first α -helix of mGM-CSF directly contact the mGM-CSF receptors. The data presented here identify in detail the qualitative and quantitative contribution of several residues in this region to this interaction. This knowledge was essential to our designing

TABLE IV

Sample ^a	$K_{0.50}$ ^b	(%CV) ^c	n ^d	% binding ^e
<i>M</i>				
GMCSF	6.2E-09	3	4	100
K14A	7.8E-09	7	1	92
K14E	7.4E-09	7	1	98
K14G	5.1E-09	12	1	140
K14P	2.0E-08	3	1	36
K14S	5.9E-09	7	1	120
K20A	4.4E-08	6	1	18
K20E	2.2E-09	15	2	180
K20F	3.0E-09	3	1	270
K20G	3.5E-08	7	1	23
K20H	6.4E-09	8	1	130
K20L	4.2E-09	6	1	110
K20M	7.1E-09	5	1	67
K20P	<5E-06	ND ^f	1	<0.1
K20Q	1.9E-08	5	1	25
K20R	4.1E-09	8	1	110
K20S	4.7E-08	6	1	11
K20V	5.5E-09	5	1	91
K20W	4.6E-09	6	1	110
K20Y	1.4E-08	5	1	37

^a Mutant proteins are identified by their native single-letter amino acid code, residue number, and amino acid substitution.

^b $K_{0.50}$ low refer to the low affinity equilibrium binding constants calculated using the Ligand program (33) from the data presented in Fig. 2A and B.

^c The percentage coefficient of variations is the standard error given by the Ligand program (33) for the $K_{0.50}$ value presented.

^d Number of LC-mGMR α competition binding assays performed.

^e Percent low affinity binding relative to values determined for wild-type mGM-CSF.

^f Not determined. Error messages from the Ligand program (33) indicated that the error was very large for this data.

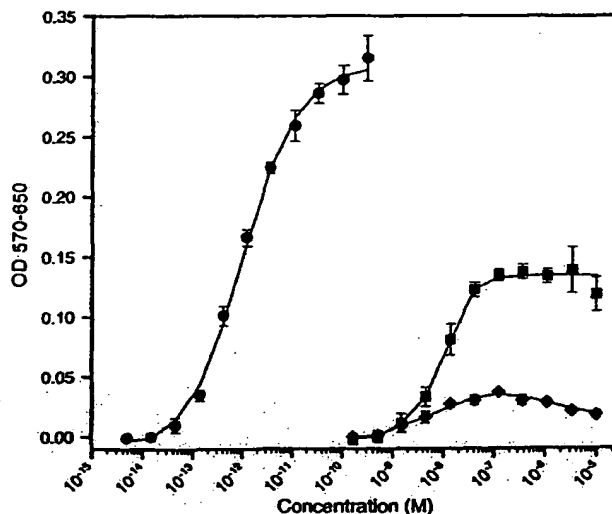


Fig. 3. Biological activity of wild-type and mutant mGM-CSF proteins. Proliferation of NFS60 cells in response to mGM-CSF (\bullet), E21K (\blacksquare), and K14E/E21K (\blacklozenge) was measured as a function of protein concentration using the MTT assay (32). Assays were performed in quadruplicate with symbol error bars indicating standard deviation.

a mGM-CSF receptor antagonist.

We have previously shown that Glu²¹ plays a pivotal role in binding to the mGM-R β in the high affinity complex. Substitution of Glu²¹ with Ala abrogates high affinity binding (16). The data presented here show that none of the 14 amino acid substitutions are tolerated at this position. Previous work concludes that Glu²¹ can be replaced with Asp without serious consequences to bioactivity (36). Analysis of the equivalent Glu²¹ position in hGM-CSF shows that Glu²¹ can be replaced with an Asp without noticeable loss in either bioactivity or

binding (18). Taken together, these data show the absolute requirement of an acidic residue at this position. All other substitutions we tested resulted in a complete loss of high affinity binding, while low affinity binding remained unaffected. These data identify Glu²¹ unequivocally as a residue that interacts with mGM-R β only. Even though Glu²¹ mediates high affinity binding, only charge reversal substitutions cause a noticeable loss in biological activity. Maintaining biological activity in the absence of high affinity binding seemingly contradicts the observation that signaling occurs through a complex of the mGM-R α and mGM-R β subunits in the presence of ligand. However, we have previously shown for one of the Glu²¹ substitution proteins p21A that this protein still can be cross-linked to the mGM-R β complex, even in the absence of any detectable high affinity binding (16). Taken together, these data indicate that the interaction of Glu²¹ with mGM-R β , although pivotal for the measured high affinity binding of mGM-CSF to its receptor, is not necessary for receptor activation. The absence of a Glu residue at position 21 apparently does not prevent mGM-R β from participating in complex formation and signaling and suggests that other mGM-CSF residues contact mGM-R β .

Studies on hGM-CSF and hIL-3, cytokines which share the β receptor, show similar results. Substitution of the homologous

acidic residues in these two cytokines also leads to a loss of high affinity binding (18, 37). However, there is a closer correlation between loss of high affinity binding and loss of activity for both hGM-CSF and hIL-3. Taken together, these data suggest that Glu²¹ in GM-CSF and the homologous acidic residue in IL-3 and presumably also IL-5 are functionally identical; this residue determines high affinity binding. However, the extent to which its absence affects the biological activity varies and presumably depends on how many other residues contribute to the interaction with GM-R β .

Our data revealed that at least 2 other residues in the first α -helix of mGM-CSF interact with mGM-R β . Substitutions at Lys¹⁴ and Lys²⁰ both disrupted high affinity binding as indicated by decreased high affinity K_d values. Lys¹⁴ substitutions exclusively reduced high affinity binding, whereas some Lys²⁰ substitutions affected high affinity binding and others low affinity binding. However, none of the substitutions at either position reduced the biological activity. The binding defects of Lys¹⁴ mutant proteins are generally somewhat smaller than Lys²⁰ defects and fell into two categories: those that were caused by defects in binding to mGM-R β and those caused by defects in binding to mGM-R α . Substitutions that reduced the binding to mGM-R α had small side chains (Ala, Ser, and Gly), suggesting that size plays a critical role at this position. Homology modeling based on the high resolution crystal structure of hGM-CSF (8) indicates the most likely location of these residues on the α -helix 1 mGM-CSF backbone (Fig. 5). Both Lys¹⁴ and Glu²¹ are located on the exterior of α -helix 1 toward the groove formed by the α -helix 1 and α -helix 3 and well situated for possible direct interaction with mGM-R β . It has been suggested that the groove between the first and third helix provides most of the GM-R β contact (38, 39). Our data on Lys²⁰ and Glu²¹ support this hypothesis. On the other hand, Lys²⁰ is at the interface of α -helix 1 and α -helix 4, which is the region thought to provide mGM-R α specific contacts. Our data support a dual role for Lys²⁰ in binding to mGM-R α and mGM-R β . It is somewhat surprising that Glu¹⁷ does not play a more important role in mGM-R β binding considering its location between Lys¹⁴ and Glu²¹ (15–17). Recent mutagenesis of the hGM-CSF equivalent position, Asn¹⁷, has confirmed that this residue contributes little to binding and bioactivity (38). Fig. 5 shows that the Glu¹⁷ side chain is somewhat removed from the groove between helix 1 and helix 3 and apparently does not contribute to binding of GM-R β .

Based on functional and structural comparison of hGM-CSF and GH, two potential receptor-binding sites on hGM-CSF have been suggested (39). Analogous to site 1 of GH (40), it was proposed that GM-CSF binds to its primary binding receptor, GM-R α , through α -helix 4. The interaction of the first α -helix of

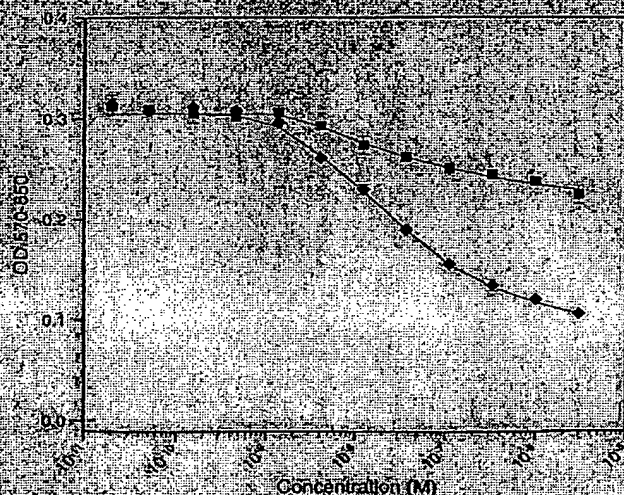
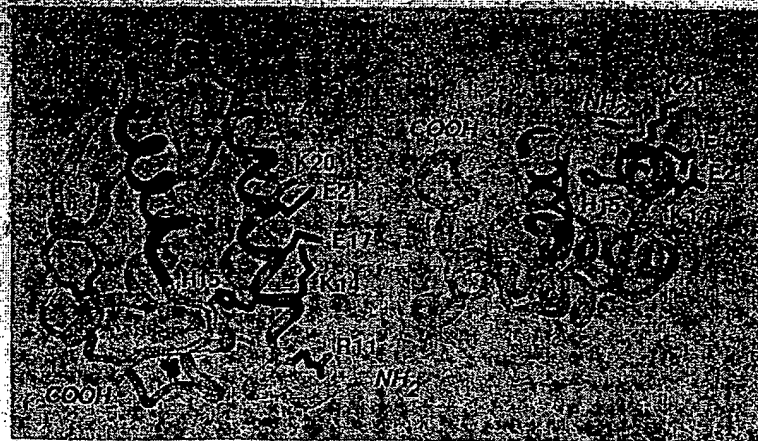


Fig. 4. Antagonism of the mGM-CSF response by mutant mGM-CSF proteins. Proliferation of NFS60 cells in response to E21K (□) and K14E/E21K (●) in the presence of 3.5×10^{-10} M mGM-CSF using the MTT assay (32). Assays were performed in quadruplicate with the symbol error bars indicating standard deviation.

Fig. 5. Two views of the mGM-CSF Ca backbone indicating important structural features and identifying specific amino acid side chains. Helix A (red) and helix D (blue) form an antiparallel helix pair and comprise the receptor binding site (38). Disulfide bonds are indicated in yellow. Amino acid residues significant to this study are colored according to the properties of their side chains: negatively charged (E), red; positively charged (K and R), blue; and aromatic (W), green.



GM-CSF with the GM-R β in the high affinity complex appears to be similar to the site II interactions of GH and GH-R. Receptor antagonists of GH were made by mutations of site II residues that prevent the second GH-R from participating in the formation of the GH-R dimer (41). We have extrapolated that finding to mGM-CSF and tested the partial agonist E21K as a receptor antagonist. E21K weakly antagonized mGM-CSF. By eliminating other mGM-R β -specific contact residues in mGM-CSF, we anticipated improved antagonistic mGM-CSF derivatives. The K14E, E21K double mutant was indeed a better antagonist than E21K. These experiments show that detailed studies which not only identify key residues involved in protein-protein interactions, but also characterize the nature of that interaction, make it possible to rationally design cytokine derivatives with altered properties.

GM-CSF, IL-3, and IL-5 all bind specific Ras but share a common R β . Identification of two R β -specific contact residues in mGM-CSF led to derivatives with antagonistic properties. This finding may have direct implications for the design of IL-3 and IL-5 antagonists. A strategy to find such molecules should focus on elimination of R β -specific contacts, in particular, the conserved acidic residue in the first α -helix of these cytokines and a second residue corresponding to mGM-CSF Lys¹⁴.

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REFERENCES

1. Metcalf, D. (1985) *Science* **229**, 16–22
2. Metcalf, D. (1986) *Blood* **67**, 257–267
3. Bazan J. F. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 6934–6938
4. Hayashida, K., Kitamura, T., Gorman, D. M., Arai, K., Yokota, T., and Miyajima, A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 9655–9659
5. Kitamura, T., Sato, N., Arai, K., and Miyajima, A. (1991) *Cell* **66**, 1165–1174
6. Park L. S., Martin, U., Sorensen, R., Lühr, S., Morrissey, P. J., Cosman, D., and Larsen, A. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 4295–4299
7. Kitamura, T., Hayashida, K., Sakamaki, K., Yokota, T., Arai, K., and Miyajima, A. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 5082–5086
8. Diederichs, K. S., Boone, T., and Karplus, P. A. (1991) *Science* **254**, 1779–1782
9. McKay D. B. (1992) *Science* **257**, 412–413
10. Smith, L. J., Redfield, C., Boyd, J., Lawrence, G. M. P., Edwards, R. G., Smith, R. A. G., and Dobson, C. M. (1992) *J. Mol. Biol.* **234**, 899–904
11. Milburn, M. V., Hassell, A. M., Lambert, M. H., Jordan, S. R., Proudfoot, A. E. I., Graber, P., and Wells, T. N. C., (1993) *Nature* **363**, 172–176
12. Pandit, J., Bohm, A., Jancarik, J., Halenbeck, R., Kotha, K., and Kim, S. (1992) *Science* **258**, 1358–1362
13. Abdel-Meguid, S. S., Shieh, H., Smith, W. W., Dayringer, H. E., Violand, R. N., and Bente, L. A. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 6434–6437
14. Shanafelt, A. B., Johnson, K. E., and Kastelein, R. A. (1991) *J. Biol. Chem.* **266**, 13804–13810
15. Shanafelt, A. B., Miyajima, A., Kitamura, T., and Kastelein, R. A. (1991) *EMBO J.* **10**, 4105–4112
16. Shanafelt, A. B. and Kastelein, R. A. (1992) *J. Biol. Chem.* **267**, 25466–25472
17. Meropol, N. J., Altmann, S. W., Shanafelt, A. B., Kastelein, R. A., Johnson, G. D., and Prystowsky, M. B. (1992) *J. Biol. Chem.* **267**, 14266–14269
18. Lopez, A. F., Shannon, M. F., Hercus, T., Nicola, N. A., Cambareri, B., Dottore, M., Layton, M. J., Eglinton, L., and Vadas, M. A. (1992) *EMBO J.* **11**, 909–916
19. Brown, C. B., Hart, C. E., Curtis, D. M., Bailey M. C., and Kaushansky, K. (1990) *J. Immunol.* **144**, 2184–2189
20. Kanakura, Y., Cannistra, S. A., Brown, C. B., Nakamura, M., Seelig, G. F., Prosser, W. W., Hawkins, J. C., Kaushansky, K., and Griffin, J. D. (1991) *Blood* **77**, 1033–1043
21. Seelig, G. F., Prosser, W. W., and Scheffler, J. E. (1994) *J. Biol. Chem.* **269**, 5548–5553
22. Messing, J. (1983) *Methods Enzymol.* **101**, 20–78
23. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol.* **154**, 367–382
24. Shanafelt, A. B., and Kastelein, R. A. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 4872–4876
25. Howard-Flanders, P., Simson, E., and Theriot, L. (1964) *Genetics* **49**, 237–246
26. Lundell, D., Greenberg, R., Alroy, Y., Condon, R., Fossetta, J. D., Gewain, K., Kastelein, R., Lunn, C. A., Reim, R., Shah, C., Van Kimmenade, A., and Narula, S. K. (1990) *J. Indust. Microbiol.* **5**, 215–228
27. Greenberg, R., Lundell, D., Alroy, Y., Bonita, S., Condon, R., Fossetta, J. D., Frommer, B., Gewain, K., Katz, M., Leibowitz, P. J., Narula, S. K., Kastelein, R., and Van Kimmenade, A. (1988) *Curr. Microb.* **17**, 321–332
28. Itoh, N., Yonehara, S., Schreurs, J., Gorman, D. M., Maruyama, K., Ishii, A., Yahara, I., Arai, K., and Miyajima, A. (1990) *Science* **247**, 324–327
29. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463–5467
30. Miyajima, A., Otsu, K., Schreurs, J., Bond, M. W., Abrams, J. S., and Arai, K. (1986) *EMBO J.* **5**, 1193–1197
31. Abrams, J. S., Roncarolo, M.-G., Yssel, H., Andersson, U., Gleich, G. J., and Silver, J. E. (1992) *Immunol. Rev.* **127**, 5–44
32. Mosmann, T. (1983) *J. Immunol. Methods* **65**, 55–63
33. Munson, P. J. (1983) *Methods Enzymol.* **92**, 543–576
34. Levitt, M. (1992) *J. Mol. Biol.* **238**, 507–533
35. Lee, C., and Subbiah, S. (1991) *J. Mol. Biol.* **217**, 373–388
36. Altmann, S. W., Johnson, G. D., and Prystowsky M. B. (1991) *J. Biol. Chem.* **266**, 5333–5341
37. Barry, S. C., Bagley, C. J., Phillips, J., Dottore, M., Cambareri, B., Moretti, P., D'Andrea, R., Goodall, G. J., Shannon, M. F., Vadas, M. A., and Lopez, A. F. (1994) *J. Biol. Chem.* **269**, 8488–8492
38. Hercus, T. R., Cambareri, B., Dottore, M., Woodcock, J., Bagley, C. J., Vadas, M. A., Shannon, M. F., and Lopez, A. F. (1994) *Blood* **83**, 3500–3508
39. Kastelein, R. A., and Shanafelt, A. B. (1993) *Oncogene* **8**, 231–236
40. de Vos, A. M., Ultsch, M., and Kossiakoff, A. A. (1991) *Science* **255**, 306–312
41. Fuh, G., Cunningham, B. C., Fukunaga, R., Nagata, S., Goeddel, D. V., and Wells, J. A. (1992) *Science* **256**, 1677–1680

Rational Design of Granulocyte-Macrophage Colony-stimulating Factor Antagonist Peptides*

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a member of the four-helix bundle family of cytokines/growth factors which exhibit several activities. It is a hematopoietic growth factor, a cytokine involved in inflammatory and immune processes, an adjunct for cancer therapy, and an anti-tumor immunomodulator. Studies of interactions between GM-CSF and its receptor and identification of small peptides presenting binding capacity to the receptor are important goals for the development of GM-CSF analogs. Here we describe the study of two cyclic peptides, 1785 and 1786, developed based on structural analysis of the GM-CSF region mimicked by anti-anti-GM-CSF recombinant antibody 23.2. These peptides were designed to structurally mimic the positions of specific residues on the B and C helices of human GM-CSF implicated in receptor binding and bioactivity. Both 1785 and 1786 were specifically recognized by polyclonal anti-GM-CSF antibody (stronger for 1786 than 1785). 1786 also competitively inhibited binding of GM-CSF to the GM-CSF receptor on HL-60 cells and demonstrated antagonist bioactivity, as shown by its reversal of GM-CSF's ability to inhibit apoptosis of the GM-CSF-dependent cell line MOTE. These studies support the role of residues on the GM-CSF B and C helices in receptor binding and bioactivity and suggest strategies for mimicking binding sites on four-helix bundle proteins with cyclic peptides.

Granulocyte-macrophage colony-stimulating factor (GM-CSF)¹ is a hematopoietic growth factor and a cytokine involved in many inflammatory and immune processes. GM-CSF activates antigen-presenting cells (monocytes, macrophages, and dendritic cells), increases major histocompatibility complex class II expression-enhancing antigen presentation, and increases macrophage anti-tumor activity (1). Recently it has

been used as an important adjunct in cancer therapy for bone marrow recovery following chemotherapy and transplantation (2). Moreover, GM-CSF induces protective immune responses against lymphoma cells if fused with a tumor-derived idiotype, eliciting tumor-specific immunity (3). GM-CSF also enhances the immunogenicity of tumor cells when expressed by them, resulting in induction of protective anti-tumor immunity, while other cytokines such as IL-2, IL-4, IL-5, IL-6, γ -interferon, or tumor necrosis factor- α are less effective (4).

The crystal structure of human GM-CSF (5–8) reveals a four-helix bundle organization similar in some respects to that described for growth hormone (9), IL-2 (10), and IL-4 (11–14). The related cytokines macrophage colony stimulating factor (15) and IL-5 are organized as dimers of four-helix bundles (16). GM-CSF activity is mediated by binding to specific cellular receptors (GM-CSFR) which belong to a recently described supergene family (17–23). The high affinity GM-CSFR is comprised of an α chain (GM-CSFR α) specific for GM-CSF (20), and a β chain (β_c), which can also associate with the IL-3 and IL-5 receptor α chains (21). The GM-CSFR α imparts specificity to the interaction with GM-CSF, and when expressed without β_c is able to bind GM-CSF, albeit with lower affinity than the heterodimeric receptor (24). The high affinity receptor (GM-CSFR α and β_c) appears to be the signal-transducing unit (25, 26), with a sequential binding of GM-CSF to GM-CSFR α followed by binding to β_c postulated.

GM-CSF and the related four-helix bundle cytokines are important targets for drug design and production of low molecular weight analogs which mimic the native ligand. Studies of ligand-receptor intermolecular interactions which help delineate their active sites should allow the development of small molecules able to mimic the larger polypeptide ligands. Such small drugs, created based on analysis of the most important binding interactions, could circumvent problems of immunogenicity, antigenicity, rapid proteolysis by serum proteolytic enzymes, short serum half-life, and low oral bioavailability, commonly presented by large polypeptides.

In prior studies, linear peptide analogs of GM-CSF were produced by dividing the human GM-CSF sequence into six peptides (27). This strategy led to the identification of two peptides with receptor binding and antagonist activity. One peptide corresponding to residues 17–31 (the A helix) inhibited high affinity receptor binding, while a second peptide corresponding to residues 54–78 (the B and C helices) inhibited low affinity receptor binding (27). This implicates these sites in intermolecular interactions with the GM-CSFR. We also have used a recombinant antibody (rAb) as a GM-CSF mimic (28). Molecular modeling of the rAb 23.2 allowed the identification of complementarity determining regions (CDRs) as sites of structural mimicry of GM-CSF, focusing attention on the CDRI

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¹ The abbreviations used are: CSF, colony-stimulating factor; GM, granulocyte-macrophage; CSFR, colony-stimulating factor receptor; IL, interleukin; rAb, recombinant antibody; TPA, 12-O-tetradecanoylphorbol-13-acetate; CDR, complementarity determining region; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

region mimicking residues on the B and C helices of GM-CSF. After synthesis and characterization of CDRI, CDRII, and CDRIII peptides, the CDRI peptide exhibited specific GM-CSF receptor binding and antagonist bioactivity (28). Thus, these studies suggest that residues on the B and C helices of GM-CSF mediate binding to the low affinity receptor (GM-CSFR α alone).

Here we describe the development of two cyclic peptide GM-CSF mimics (1785 and 1786) obtained from structural analysis of the GM-CSF region mimicked by rAb 23.2 (28). Cysteines were introduced in the peptide structures at the amino and carboxyl termini to allow cyclization. The cyclized peptides were specifically bound by polyclonal anti-GM-CSF antibody (stronger for 1786 than for 1785). Moreover, 1786 competes with GM-CSF for binding to the GM-CSF receptor present on HL-60 cells and reverses GM-CSF's prevention of apoptosis of MOTE cells. Thus, 1786 represents a structurally designed biological and receptor antagonist of GM-CSF.

MATERIALS AND METHODS

Design of Peptides 1785 and 1786—1785 and 1786 were the result of the comparison of the GM-CSF mimic rAb 23.2 molecular models and its individual CDR sequences with the human GM-CSF structure (28). Despite the weak primary sequence similarity shown by 23.2 with GM-CSF, structural similarity was suggested centered on the B (residues 54–61) and C (residues 77–83) helices of GM-CSF and the 23.2 CDRI region (28). Important residues in the GM-CSF structure mimicked by similar residues on 23.2 were postulated to be: Thr-57, Glu-60, Lys-63, Lys-74, Thr-78, Ser-82, and Lys-85. Based on the ability of a predicted reverse turn structure (the 23.2 CDRI) to functionally mimic this site on GM-CSF, two distinct reverse turns were designed using the MacInmad program (Molecular Applications Group, Stanford, CA). Peptide 1786 was designed beginning at Thr-57 and proceeding up the exposed residues on the B helix (Glu-60 and Lys-63), then continuing in the reverse orientation on the C helix (Lys-74, Thr-78, Ser-82, and Lys-85). Glycine or alanine residues were introduced to orient the predicted contact residues on the same face of the reverse turn. Additional Gly residues were added at the amino and carboxyl termini to appropriately position Cys residues for cyclization by disulfide bridge formation. The 1785 peptide was designed according to the same principles, but beginning with Lys-85 on the C helix and proceeding in the opposite orientation. The sequences of these peptides and their predicted structures in comparison with the GM-CSF structure is shown in Fig. 1.

Preparation of Cyclic Peptides—The two peptides were synthesized by solid phase methods, deprotected, and released from the resin by anhydrous HF as described previously (29–32) by Macromolecular Resources at Colorado State University (C. Miles). Peptides (containing cysteine residues as terminal amino acids) were oxidized dissolving them at 0.5 mg/ml in 50 mM NH_4HCO_3 , pH 8.0, and stirring them overnight exposed to the air at room temperature. The extent of oxidation was estimated by Ellman determination after this procedure.

Determination of Free Sulfhydryls in Peptides (Ellman Determination)—20, 40, 80, and 160 μl of peptides at 0.5 mg/ml in 50 mM NH_4HCO_3 , pH 8.0, were added to 10 mM Na_2HPO_4 , pH 7.0, for a final volume of 1 ml. 6 μl of 4 mg/ml 5,5'-dithio-bis(2-nitrobenzoic acid) (Sigma) in 50 mM Na_2HPO_4 , pH 7.0, were then added, and the reaction mixtures were kept at room temperature for 10 min. The percentage of sulfhydryls was determined from the absorbance at 420 nm, using the formula: $(100 \times A_{420} \times M)/(13600 \times \text{mg/ml})$.

Peptide Characterization—The formation of peptide intrachain disulfide bridge versus interchain bridges was estimated by mass spectrometry analysis performed at the Protein Chemistry Laboratory of the University of Pennsylvania School of Medicine (J. Lambris). This indicated >90% monomers of the oxidized peptides.

Enzyme-linked Immunosorbent Assay (ELISA)—ELISA was performed with polystyrene plates (Dynatech Laboratories Inc., Chantilly, VA). The peptides 1785, 1786, and a control peptide (Cys-Thr-Tyr-Arg-Tyr-Pro-Leu-Glu-Leu-Asp-Thr-Ala-Asn-Asn-Arg) were dissolved in 50 mM NH_4HCO_3 at 120, 90, 60, and 30 $\mu\text{g/ml}$ and 50 μl of each dilution were used to coat the wells in duplicate by evaporation overnight at 37 °C. As positive controls wells were coated with 50 μl of 1 $\mu\text{g/ml}$ GM-CSF in 0.1 M NaHCO_3 overnight at 4 °C. The wells were then washed with PBS, 0.05% Tween 20 (PBST), and blocked for 1 h at 37 °C with PBS, 0.05% Tween, 2% bovine serum albumin (PBSTB). After

washing with PBST, 50 $\mu\text{l/well}$ of primary antibody (polyclonal antibody against GM-CSF previously described (28, 33)) and preimmune serum (normal mouse serum) as a negative control were added at 1:1,000, 1:10,000, 1:100,000, and 1:1,000,000 dilutions in PBSTB; and the plate was incubated for 1 h at 37 °C. The plate was washed, and 200 μl of secondary antibody, goat anti-mouse conjugated to horseradish peroxidase (Sigma) diluted 1:3500 in PBSTB was added to the wells, and the plate was incubated for 1 h at 37 °C. After washing, the color reagent 3,3',5,5'-tetramethyl-benzidine dihydrochloride (Sigma) 0.1 mg/ml was added at 100 $\mu\text{l/well}$ and, after 10 min of incubation at 37 °C, the color reaction was stopped with 20 $\mu\text{l/well}$ of 2 N H_2SO_4 , and the absorbance at 450 nm was detected using the plate reader MR 5000 (Dynatech Laboratories Inc., Chantilly, VA). Values were reported subtracting the absorbance measured for uncoated wells from the absorbance of peptide-coated wells (34).

Radioreceptor Binding Assay—Binding of 1785 and 1786 to the GM-CSF receptor present on HL60 cells was analyzed by a competitive radioreceptor assay modified from previously reported protocols (20, 35). Briefly, HL60 (from ATCC) were grown in RPMI 1640 with 10% fetal calf serum, L-glutamine, oxalate, pyruvate, insulin, essential amino acids, and nonessential amino acids. 10^6 cells were washed twice in RPMI 1640, 10 mM Hepes, pH 7.4, 10% fetal calf serum (binding buffer), centrifuged, and incubated with different dilutions of peptides 1785, 1786, and control peptide (for final concentrations of 500, 250, 125, 62.5, and 31.25 $\mu\text{g/ml}$) for 1 h at room temperature. ^{125}I -GM-CSF (118 $\mu\text{Ci}/\mu\text{g}$, Dupont NEN) was then added to the reaction mixtures at a final concentration of 0.5 nM (for total GM-CSF bound) or a mixture of radioiodinated (0.5 nM), and cold GM-CSF (at the saturating concentration of 50 nM) was added (for nonspecific binding) and incubated at room temperature for 1 h. The mixture was then layered over 500 μl of chilled fetal calf serum and centrifuged, and the counts/min bound determined in an LKB gamma counter. Specific binding was determined by subtracting the nonspecific counts/min bound from the total counts/min bound. Scatchard analysis revealed that, at this concentration, predominantly low affinity sites (2.9 nM) were measured (27) (data not shown). Based on the EC_{50} achieved by peptide, the K_i was calculated by the method of Cheng and Prusoff (36).

Inhibition of Apoptosis—The assay was performed in a 24-well polystyrene plate (Corning, Costar Corp., Cambridge, MA), using MOTE cells (from R. Zollner, Genetics Institute, Cambridge MA), grown in RPMI 1640, 10% fetal calf serum, L-glutamine, oxalate, pyruvate, insulin, nonessential amino acids, essential amino acids, penicillin/streptomycin, and 20% U87 supernatant (containing GM-CSF as a growth factor). The peptides were added at different dilutions to the wells (final concentrations of 160, 80, 40, and 0 $\mu\text{g/ml}$). After sterilization of the plates under UV light for 40 min, fixed amounts of sterile GM-CSF (200 pM), TPA (12-O-tetradecanoylphorbol-13-acetate (Sigma), 4 nM), and U87 supernatant (5%) were added separately to all the different concentrations of peptide: 250 μl of cell suspension at 10^6 cells/ml, previously washed with the medium without U87 supernatant and resuspended in the same medium, were added to each well, reaching a final concentration 5×10^5 cells/ml in a total volume of 500 μl . After 24 h of growth, cells were lysed and DNA degradation detected both by an agarose gel run and by the use of "Cell Death Detection-ELISA" kit (Boehringer Mannheim).

For the agarose evaluation, 350 μl of 5×10^5 cells/ml were washed, added to 20 μl of lysis buffer (10 mM EDTA, 50 mM Tris HCl pH 8.0, 0.5% N-lauroylsarcosine sodium salt (Sarkosyl), 0.5 mg/ml proteinase K) and incubated for 1 h at 50 °C. After addition of 10 μl of 0.45 mg/ml RNase and incubation at 50 °C for 1 h, the samples were mixed with 10 μl of 10 mM EDTA, pH 8.0, 0.03% bromophenol blue, 1% Nue Sieve GTC agarose (FMC BioProducts, Rockland, ME), heated at 70 °C for 10 min, loaded into a 1.2% agarose gel and run for 1 h at 100 V using TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA pH 8.0). The gel was stained with ethidium bromide (Sigma) and photographed under ultraviolet light.

Mono- and oligonucleosome fragments present in the cytoplasmic fraction of cell lysates were detected following the protocol for "Cell Death Detection ELISA" kit. Briefly, the microtiter plate was coated with anti-histone solution and, after incubation with a 1:10 dilution of the lysate derived from 2.5×10^6 cells, DNA was detected by the anti-DNA-peroxidase system according to the kit instructions, with color development read at 405 nm.

RESULTS

Peptide Design—The two peptides were synthesized according to the sequences reported in Fig. 1. The postulated contact

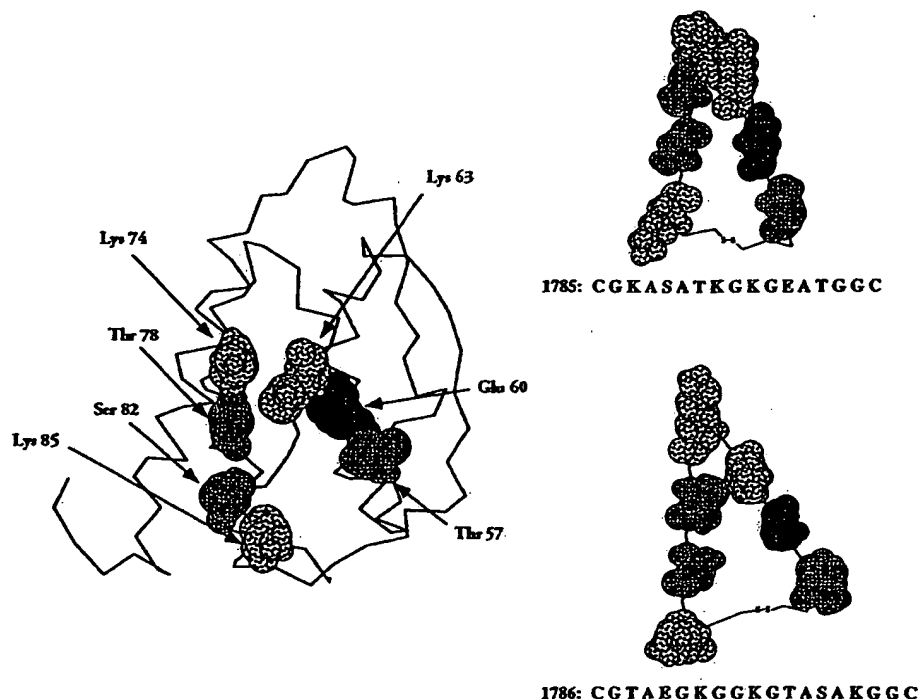


FIG. 1. Development of 1785 and 1786 peptides. The structure of GM-CSF (left) was determined from coordinates derived from the crystal structure (J. M. LaLonde, K. Swaminathan and D. Voet, manuscript in preparation), displayed on the MacImdad program (Molecular Applications Group, Palo Alto, CA) on a Macintosh Quadra 950 computer. The critical residues of 54–61 region of B helix and of 77–83 region on C helix are reported. These residues are introduced in 1785 (upper right) and 1786 (lower right) sequences together with glycines, alanines, and cysteines (for peptide cyclization). Peptide tridimensional structures are also reported.

residues on the GM-CSF B and C helices (regions involved in the interaction with GM-CSFR α) were introduced into the peptides in two different orientations ("up" the B and "down" the C helices for 1786, with the opposite orientation for 1785). The design incorporated reverse turn structures together with appropriate spacer residues and cysteines at the amino and carboxyl termini, which allowed the development of cyclic forms.

Peptide Cyclization—The procedure followed for peptide cyclization was oxidation of the terminal sulfhydryls with intrachain disulfide bond formation. Ellman determination indicated that only 4.7 and 1.8% of free sulfhydryls were still present in the oxidized forms of 1785 and 1786, respectively, confirming near complete oxidation of the peptides.

Mass spectrometry analysis was performed on the oxidized peptides to confirm that oxidation had resulted in intrachain disulfide bond formation, as opposed to formation of oligomers. The mass spectrometry study showed that >90% of the oxidized 1785 peptide was represented by a peak at molecular mass 1514, with the theoretical molecular mass for 1785 being 1511 daltons. Similarly, >90% of the 1786 peptide was seen as a peak at molecular mass 1637, the theoretical molecular mass being 1639. Thus, both of the oxidized peptides were >90% in the monomeric form, with only trace contamination by oligomers (dimers and trimers).

Recognition of Peptides by Polyclonal Antibody against GM-CSF—The ability of these peptides to mimic GM-CSF was initially evaluated by its recognition by polyclonal antibody against GM-CSF in an ELISA (Fig. 2). Neither peptide showed any specific binding by the preimmune serum (normal mouse serum), indicating lack of nonspecific binding. Both peptides 1785 and 1786 were specifically bound by polyclonal antibody against GM-CSF, with the titer higher for 1786 than that for 1785. The control peptide was not bound by the anti-GM-CSF, further supporting specific recognition of the peptide mimics. This supports structural mimicry of GM-CSF by the peptides 1785 and 1786.

Peptide Binding to the GM-CSF Receptor—The ability of the peptides to bind the GM-CSF receptor was evaluated by their ability to compete with GM-CSF for binding to the GM-CSF receptor present on HL-60 cells as evaluated by a radioreceptor assay. Peptides were preincubated with HL-60 cells prior to the addition of 125 I-GM-CSF, and specific binding was determined by carrying out identical reactions in the presence of excess of unlabeled GM-CSF. Fig. 3 reports the typical results obtained with 1786, 1785, and control peptides. While 1785 and control peptides failed to show any specific inhibitory activity, 1786 inhibited GM-CSF binding to its receptor in a dose-dependent manner, with 50% inhibition achieved at ~ 500 μ g/ml. 1786 therefore antagonizes GM-CSF binding to its receptor, indicating binding of this peptide to the GM-CSFR on HL-60 cells. Scatchard analysis of GM-CSF binding to HL-60 cells reveals high affinity (46 pM) and low affinity (2.9 nM) sites for GM-CSF binding (20, 35). Under the conditions of the assays here, predominately low affinity sites were measured (27) (data not shown). Based on the low affinity K_d , calculation of the K_i for peptide using the method of Cheng and Prusoff (36) gives a value of 270 μ M.

Bioactivity of Peptides—GM-CSF bioactivity can be evaluated by its ability to inhibit spontaneous apoptosis of the GM-CSF-dependent cell line MO7E (37, 38). This assay is of particular utility as it can also be applied to stimuli which inhibit apoptosis independent of signaling through the GM-CSF receptor (37). To analyze the bioactivity shown by the 1786 and 1785 peptides, their capacity to interfere with GM-CSF's ability to prevent apoptosis in MO7E cells was assayed. Apoptosis was evaluated both by agarose gel electrophoresis of total cellular DNA and by a specific ELISA assay. In addition to GM-CSF, two other stimuli were evaluated: phorbol ester (TPA), which inhibits apoptosis in a receptor-independent fashion, and GM-CSF containing U87 cell supernatant.

Both the agarose gel and the ELISA results (Fig. 4) indicated clear antagonist activity for the 1786 peptide, with reversal of

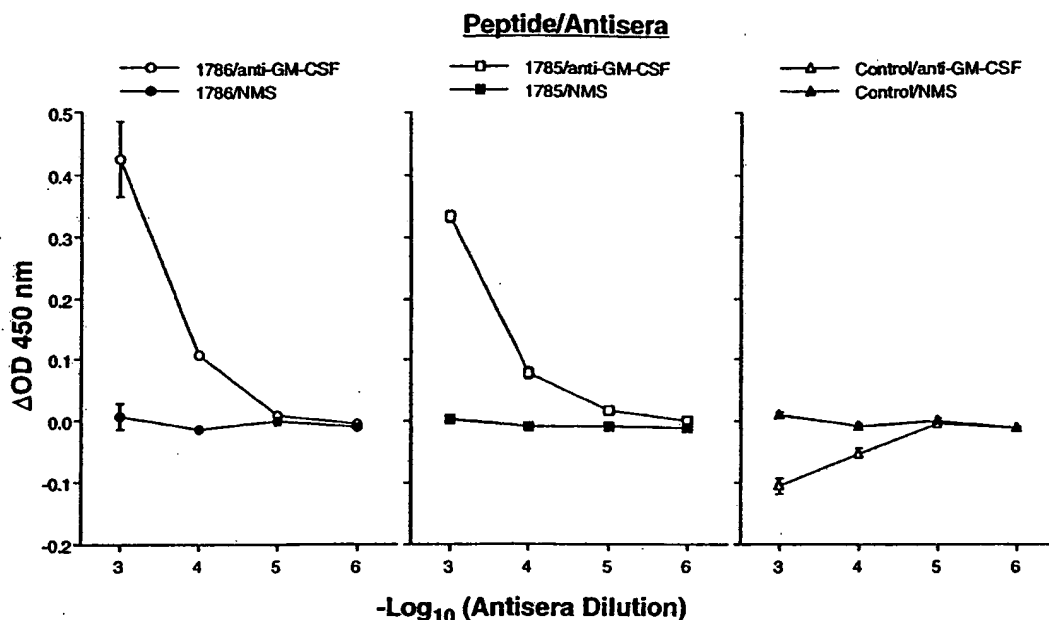


FIG. 2. Binding of polyclonal Ab against GM-CSF to 1786 and 1785 peptides. Binding was performed by ELISA assay as described under "Material and Methods." The graphs shown are referred to the case of peptides at 1.5 $\mu\text{g}/\text{well}$. Similar results were obtained with 3, 4.5, and 6 $\mu\text{g}/\text{well}$. Binding of 1786, 1785, and control peptides both to anti-GM-CSF polyclonal antibody and to preimmunization serum (normal mouse serum, NMS) are reported. The values are obtained subtracting the A_{450} of wells without peptides from the A_{450} of wells with peptides at different concentrations. The mean \pm S.D. of duplicate wells is shown for decreasing amounts of polyclonal anti-GM-CSF antibody.

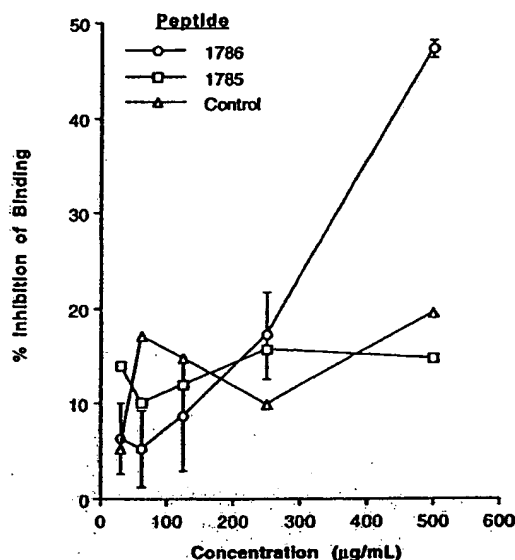


FIG. 3. Inhibition of ^{125}I -GM-CSF binding to HL-60 cells by peptides. The radioreceptor assay was performed as reported under "Materials and Methods," using 10^5 cells/test. The specific proportion of count/min bound was determined subtracting the proportion of counts/min bound under identical conditions in the presence of saturating amounts of unlabeled GM-CSF (50 nM). The percent inhibition of binding of 1786, 1785, and control peptide is reported versus increasing amounts of peptides together with the S.D. of duplicate tests.

GM-CSF's prevention of apoptosis. Increasing the amount of 1786 in presence of GM-CSF increased the amount of apoptosis seen (IC_{50} of $\sim 85 \mu\text{M}$). When incubated with the cells in medium alone, the 1786 peptide did not prevent DNA degradation, excluding any agonist activity by the peptide. The same peptide, in the presence of U87 cell supernatant, presented the same type of dose-dependent behavior in increasing apoptosis as shown in presence of GM-CSF (IC_{50} of $\sim 65 \mu\text{M}$). The 1786 effect was not seen in the presence of TPA which prevents apoptosis in a receptor independent fashion, indicating that the

antagonist activity was GM-CSF receptor-dependent. In contrast, the 1785 peptide did not demonstrate agonist or antagonist activity in these apoptosis assays. This indicates that 1786, which inhibits GM-CSF receptor binding, has a similarly specific GM-CSF receptor-dependent antagonist bioactivity.

DISCUSSION

The interaction of GM-CSF with its receptor has been the subject of intense investigation. Prior studies with GM-CSF mutants indicated that residues on the first (A) helix of GM-CSF are involved in the binding to high affinity receptor (the GM-CSFR $\alpha\beta_c$ complex) but not to low affinity receptor (GM-CSFR α alone) (24, 39, 40). This is illustrated most strikingly by studies using mutants of residue Glu-21 of GM-CSF, which inhibit binding of GM-CSF to the low affinity receptor, but display little activity in inhibiting binding to the high affinity receptor (39, 41, 42). Based on these experiments, it has been proposed that the first α helix of GM-CSF is responsible for binding to β_c (40).

Murine and human GM-CSF display species specificity and are not cross-reactive. As substitutions are scattered throughout these molecules, it was possible to swap regions of murine and human GM-CSF to locate sites critical for receptor interaction (35). These studies indicated a critical role for amino acids 21–31 (A helix) and 77–94 (including the C helix) in mediating the activity of human GM-CSF, suggesting that the second site may be involved in binding to the GM-CSFR α . Additional mutagenesis studies (42–45), mapping of neutralizing monoclonal antibodies (46–50), and synthetic peptide studies (47, 51, 52) suggest other potential interaction sites. Thus, in spite of considerable study, the GM-CSFR α interaction site(s) on GM-CSF remain incompletely characterized.

In our group use of synthetic peptides, anti-peptide antisera, and neutralizing monoclonal antibody to map epitopes on GM-CSF important for bioactivity have led to several conclusions: a peptide corresponding to residues 17–31 of the A helix, as well as antibodies against this peptide, are able to inhibit GM-CSF dependent cellular proliferation; the 17–31 peptide also inhibits GM-CSF binding to the high affinity receptor but not to the

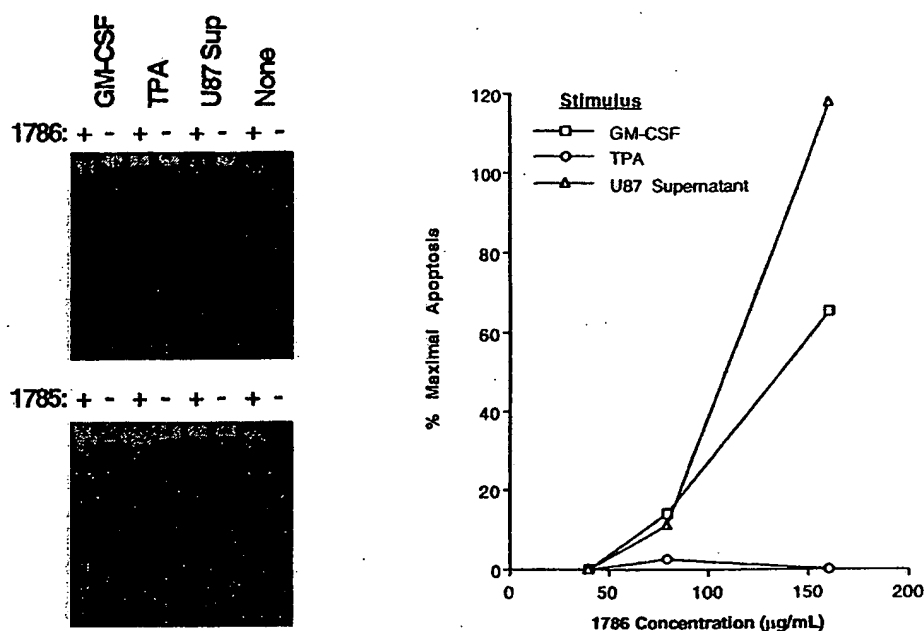


FIG. 4. Inhibition of GM-CSF's prevention of apoptosis by peptides. Apoptosis was evaluated both by running cell lysate in an agarose gel (left, reported only for the case of peptides at 160 μ g/ml) and by determining mono- and oligonucleosomes with an ELISA kit (right, reported only for the significant 1786 peptide). The assays were performed as indicated under "Materials and Methods." Lysates from cells incubated with or without peptides in presence or absence of factors preventing apoptosis (GM-CSF, TPA, U87 supernatant) were loaded into gel (left) or analyzed by ELISA reporting the percentage of maximal apoptosis, as referred to the absence of any reagent preventing apoptosis (right).

low affinity receptor; a peptide corresponding to residues 54–78 overlapping the B and C helices is recognized by two neutralizing monoclonal antibodies to GM-CSF and exhibits antagonist bioactivity (27). This suggests a model of receptor interaction where residues on the B and C helices of GM-CSF, the opposite face of the A helix, are involved in interactions with GM-CSFR α , while residues on the A helix mediate binding to β_c (27). This model is supported by analysis of a rAb mimic of GM-CSF (23.2) as well as a peptide derived from the CDRI sequence of the rAb 23.2. The CDRI peptide and the rAb were shown to exhibit structural similarity to residues on the GM-CSF B and C helices; both the peptide and the rAb mimic were bound by neutralizing anti-GM-CSF monoclonal antibody 126.213 and exhibited biological and/or receptor antagonist activity (28, 33).

The purpose of this study was to further test this model by developing additional peptides which mimic the position of specific residues on the GM-CSF B and C helices, and evaluating them for receptor binding and biological activity. The structure of the two peptides discussed in this report derived from our prior studies, with 1785 and 1786 designed to structurally mimic potential contact residues on the GM-CSF B (residues 54–61) and C (residues 77–83) helices. The two peptides were synthesized with cysteines at both the amino and carboxyl termini in order to develop cyclic forms, thereby constraining the conformations of the peptides and providing more accurate mimicry of the B and C helical face of GM-CSF. These peptides also allowed us to evaluate whether reverse turn peptide mimics, such as those developed from the rAb 23.2 CDRI sequence, could be developed from simple structural considerations, obviating the need to develop them by library screening or antibody mimicry.

The cyclic peptides were easily prepared by oxidation overnight, reaching almost 100% oxidation and 90% yield (only traces of dimer and trimer were detected by mass spectrometry analysis). The cyclized monomer peptides were therefore used in binding tests to polyclonal antibody against GM-CSF and to GM-CSF receptor present on HL60 cells. In both cases peptide

1786 showed good binding capacity, displaying competitive behavior toward GM-CSF in the radioreceptor assay. On the other hand, peptide 1785 demonstrated a lower binding affinity to polyclonal anti-GM-CSF antiserum and complete lack of interaction with the GM-CSF receptor. In order to establish their bioactivity, the peptides were assayed in an apoptosis assay. GM-CSF is known to prevent apoptosis of MO7E cells (37, 38). These cells were incubated, in presence or absence of different concentrations of peptides, with fixed amounts of GM-CSF or U87 supernatant (a source of GM-CSF). TPA, an agent which also prevents apoptosis but via a different mechanism not involving the GM-CSF receptor, allowed the specificity of the reaction to be evaluated (37). Peptide 1786, but not 1785 or control peptides, displayed biological antagonist activity: increasing the amount of peptide 1786 resulted in an increase in apoptosis in response to GM-CSF or U87 supernatant, while no effect was seen in the presence of TPA.

The IC_{50} for peptide 1786 in the apoptosis assay was similar for both GM-CSF and U87 supernatant (65–85 μ M). This is somewhat smaller than the calculated K_i for peptide inhibition of binding to low affinity receptor sites on HL-60 cells (270 μ M). However, the low affinity sites do not appear to mediate bioactivity, while the high affinity sites do (21, 39). Interestingly, if a similar EC_{50} is assumed for the high affinity sites, the calculated K_i for peptide 1786 in the binding assay is 59 μ M (36), much closer to the IC_{50} observed in the apoptosis assay. This supports the role of high affinity sites in mediating bioactivity.

Based on these studies, peptide 1786 represents a receptor antagonist of GM-CSF, supporting our conclusions from molecular-structural analysis utilizing recombinant antibodies (28) for the identification of residues critical for bioactivity. Moreover, these studies suggest that similar peptide mimics can be designed based on structural information derived from knowledge of potential contact residues. The ability to design such mimics may be readily extended to other systems where sufficient structural and biological information is available to delineate potential contact residues. This should allow for the analysis of potential contact residues on novel backbones as

well as the rational design of receptor antagonists with potential clinical utility.

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REFERENCES

- Welsbort, R., and Golde, D. (1989) *Hematol. Oncol. Clin. North Am.* 3, 401–409
- Antman, K. H. (1993) *Eur. J. Cancer* 3, S2–S6
- Tao, M.-H., and Levy, R. (1993) *Nature* 362, 755–758
- Dranoff, G., Jaffee, E., Lazenby, A., Columbick, P., Levitsky, H., Brose, K., Jackson, V., Hamada, H., Pardoll, D., and Mulligan, R. C. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 3539–3543
- Diederichs, K., Boone, T., and Karpus, P. A. (1991) *Science* 254, 1779–1782
- Diederichs, K., Jacques, S., Boone, T., and Karpus, P. A. (1991) *J. Mol. Biol.* 221, 55–60
- LaLonde, J. M., Hanna, L. S., Rattoballi, R., Berman, H. M., and Voet, D. (1989) *J. Mol. Biol.* 205, 783–785
- Walter, M. R., Cook, W. J., Eallick, S. E., Nagabhushan, T. L., Trotta, P. P., and Bugg, C. E. (1992) *J. Mol. Biol.* 224, 1075–1085
- deVos, A. M., Ultsch, M., and Kossiakoff, A. A. (1992) *Science* 255, 306–312
- Brandhuber, B. J., Boone, T., Kenney, W. C., and McKay, D. B. (1987) *Science* 238, 1707–1709
- Garrett, D. S., Powers, R., March, C. J., Frieden, E. A., Clore, G. M., and Gronenborn, A. M. (1992) *Biochemistry* 31, 4347–4353
- Powers, R., Garrett, D. S., March, C. J., Frieden, E. A., Gronenborn, A. M., and Clore, G. M. (1993) *Biochemistry* 32, 6744–6762
- Redfield, C., Boyd, J., Smith, L. J., Smith, R. A., and Dobson, C. M. (1992) *Biochemistry* 31, 10431–10437
- Smith, L. J., Redfield, C., Boyd, J., Lawrence, G. M., Edwards, R. G., Smith, R. A., and Dobson, C. M. (1992) *J. Mol. Biol.* 224, 899–904
- Pandit, J., Bohm, A., Jancarik, J., Halenbeck, R., Koths, K., and Kim, S. H. (1992) *Science* 258, 1358–13562
- Milburn, M. V., Hassell, A. M., Lambert, M. H., Jordan, S. R., Proudfoot, A. E., Graber, P., and Wells, T. N. (1993) *Nature* 363, 172–1726
- Chiba, S., Tojo, A., Kitamura, T., Urabe, A., Miyazono, K., and Takaku, F. (1990) *Leukemia* 4, 29–36
- Cannistra, S. A., Groshek, P., Garlick, R., Miller, J., and Griffin, J. D. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 93–97
- DiPersio, J., Billing, P., Kaufman, S., Eghtesady, P., Williams, R. E., and Gasson, J. C. (1988) *J. Biol. Chem.* 263, 1834–1841
- Gearing, D. P., King, J. A., Gough, N. M., and Nicola, N. A. (1989) *EMBO J.* 8, 3667–3676
- Hayashida, K., Kitamura, T., Gorman, D. M., Arai, K.-I., Yokota, T., and Miyajima, A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 9655–9659
- Park, L., Friend, D., Gillis, S., and Urdal, D. (1986) *J. Biol. Chem.* 261, 4177–4183
- Onetto-Pothier, N., Aumont, N., Haman, A., Bigras, C., Wong, G. G., Clark, S. C., De Lean, A., and Hoang, T. (1990) *Blood* 75, 59–66
- Shanafelt, A. B., and Kastelein, R. A. (1992) *J. Biol. Chem.* 267, 25466–25472
- Yokota, T., Watanabe, S., Mui, A. L., Muto, A., Miyajima, A., and Arai, K. (1993) *Leukemia* 7, S102–S107
- Sakamaki, K., Miyajima, I., Kitamura, T., and Miyajima, A. (1992) *EMBO J.* 11, 3541–3549
- VonFeldt, J. M., Monfardini, C., Fish, S., Rosenbaum, H., Kieber-Emmons, T., Williams, R. M., Kahn, S. A., Weiner, D. B., and Williams, W. V. (1995) *Peptide Res* 8, 20–32
- Monfardini, C., Kieber-Emmons, T., VonFeldt, J. M., O'Malley, B., Rosenbaum, H., Godillot, A. P., Kaushansky, K., Brown, C. B., Voet, D., McCallus, D. E., Weiner, D. B., and Williams, W. V. (1995) *J. Biol. Chem.* 270, 6628–6638
- Williams, W., Guy, H., Rubin, D., Robey, F., Myers, J., Kieber-Emmons, T., Weiner, D., and Greene, M. (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85, 6488–6492
- Williams, W., Moss, D., Kieber-Emmons, T., Cohen, J., Myers, J., Weiner, D., and Greene, M. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 5537–5541
- Williams, W., Kieber-Emmons, T., Weiner, D., Rubin, D., and Greene, M. (1991) *J. Biol. Chem.* 266, 9241–9250
- Williams, W., Kieber-Emmons, T., VonFeldt, J., Greene, M. I., and Weiner, D. (1991) *J. Biol. Chem.* 266, 5182–5190
- VonFeldt, J. M., Monfardini, C., Kieber-Emmons, T., Voet, D., Weiner, D. B., and Williams, W. V. (1994) *Immunol. Res.* 13, 96–109
- Ugen, K. E., Goedert, J. J., Boyer, J., Refaelli, Y., Frank, I., Williams, W. V., Willoughby, A., Landesman, S., Mendez, H., Rubinstein, A., Kieber-Emmons, T., and Weiner, D. B. (1992) *J. Clin. Invest.* 89, 1923–1930
- Kaushansky, K., Shoemaker, S., Alfaro, S., and Brown, C. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 1213–1217
- Cheng, Y. C., and Prusoff, W. H. (1973) *Biochem. Pharmacol.* 22, 3099–3108
- Rajotte, D., Haddad, P., Haman, A., Cragoe, E. J., Jr., and Hoang, T. (1992) *J. Biol. Chem.* 267, 9980–9987
- Horie, M., and Broxmeyer, H. E. (1995) *Exp. Hematol.* 23, 168–173
- Lopez, A. F., Shannon, M. F., Hercus, T., Nicola, N. A., Cambareri, B., Dottore, M., Layton, M. J., Eglinton, L., and Vadas, M. A. (1992) *EMBO J.* 11, 909–916
- Shanafelt, A. B., Miyajima, A., Kitamura, T., and Kastelein, R. A. (1991) *EMBO J.* 10, 4105–4112
- Hercus, T. R., Bagley, C. J., Cambareri, B., Dottore, M., Woodcock, J. M., Vadas, M. A., Shannon, M. F., and Lopez, A. F. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 5838–5842
- Hercus, T. R., Cambareri, B., Dottore, M., Woodcock, J., Bagley, C. J., Vadas, M. A., Shannon, M. F., and Lopez, A. F. (1994) *Blood* 83, 3500–3508
- Gough, N., Grail, D., Gearing, D., and Metcalf, D. (1987) *Eur. J. Biochem.* 169, 353–358
- Shanafelt, A. B., and Kastelein, R. A. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 4872–4876
- Altman, S. W., Johnson, G. D., and Prystowsky, M. B. (1991) *J. Biol. Chem.* 266, 5333–5341
- Brown, C. B., Hart, C. E., Curtis, D. M., Bailey, M. C., and Kaushansky, K. (1990) *J. Immunol.* 144, 2184–2189
- Kanakura, Y., Cannistra, S. A., Brown, C. B., Nakamura, M., Seelig, G. F., Prossie, W. W., Hawkins, J. C., Kaushansky, K., and Griffin, J. D. (1991) *Blood* 77, 1033–1043
- Nice, E., Dempsey, P., Layton, J., Morstyn, G., Cui, D. F., Simpson, R., Fabri, L., and Burgess, A. (1990) *Growth Factors* 3, 159–169
- Seelig, G., Prossie, W., Scheffler, J., Nagabhushan, T., and Trotta, P. (1990) *J. Cell. Biochem.* 14C, 246
- Brown, C. B., Pihl, C. E., and Kaushansky, K. (1994) *Eur. J. Biochem.* 225, 873–880
- Greenfield, R. S., Braslawsky, G. R., Kadow, K. F., Spitalny, G. L., Chace, D., Bull, C. O., and Bursucker, I. (1993) *J. Immunol.* 150, 5241–5251
- Clark-Lewis, I., Lopez, A. F., To, L. B., Vadas, M. A., Schrader, J. W., Hood, L. E., and Kent, S. B. H. (1988) *J. Immunol.* 141, 881–889

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Residue 21 of human granulocyte-macrophage colony-stimulating factor is critical for biological activity and for high but not low affinity binding

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The functional role of the predicted first α -helix of human granulocyte-macrophage colony-stimulating factor (GM-CSF) was analysed by site-directed mutagenesis and multiple biological and receptor binding assays. Initial deletion mutagenesis pointed to residues 20 and 21 being critical. Substitution mutagenesis showed that by altering Gln20 to Ala full GM-CSF activity was retained but that by altering Glu21 for Ala GM-CSF activity and high affinity receptor binding were decreased. Substitution of different amino acids for Glu21 showed that there was a hierarchy in the ability to stimulate the various biological activities of GM-CSF with the order of potency being Asp21 > Ser21 > Ala21 > Gln21 > Lys21 = Arg21. To distinguish whether position 21 was important for GM-CSF binding to high or low affinity receptors, GM-CSF (Arg21) was used as a competitor for [¹²⁵I]GM-CSF binding to monocytes that express both types of receptor. GM-CSF (Arg21) exhibited a greatly reduced capacity to compete for binding to high affinity receptors, however, it competed fully for [¹²⁵I]GM-CSF binding to low affinity receptors. Furthermore, GM-CSF (Arg21) was equipotent with wild-type GM-CSF in binding to the cloned low affinity α -chain of the GM-CSF receptor. These results show that (i) this position is critical for high affinity but not for low affinity GM-CSF receptor binding thus defining two functional parts of the GM-CSF molecule; (ii) position 21 of GM-CSF is critical for multiple functions of GM-CSF; and (iii) stimulation of proliferation and mature cell function by GM-CSF are mediated through high affinity receptors.

Key words: growth factors/haemopoiesis/mutagenesis/structure – function/receptors

Introduction

Human (h) granulocyte-macrophage (GM) colony-stimulating factor (CSF) is a multi-potential growth factor capable of stimulating several haemopoietic cell lineages such as the neutrophilic, eosinophilic, monocytic and megakaryocytic series (Sieff *et al.*, 1985; Metcalf *et al.*, 1986). In addition GM-CSF is also able to stimulate the function of the differentiated progeny enhancing the effector functions of neutrophils and eosinophils (Vadas *et al.*, 1983;

Gasson *et al.*, 1984; Lopez *et al.*, 1986) and the capacity of monocytes to kill tumour cells (Grabstein *et al.*, 1986) and adhere to various surfaces (Gamble *et al.*, 1989; Elliott *et al.*, 1990). Because of this pleiotropic effect GM-CSF has been used *in vivo* where it has been shown to increase the granulocyte counts in AIDS patients (Groopman *et al.*, 1987), accelerate bone marrow reconstitution following chemotherapy (Antman *et al.*, 1988) and enhance the effector function of circulating neutrophils (Baldwin *et al.*, 1988). In addition to normal haemopoietic cells, certain tumour cell lines have also been shown to respond to GM-CSF by proliferating *in vitro* (Dedhar *et al.*, 1988; Berdel *et al.*, 1989).

The human GM-CSF receptor has now been cloned and shown to comprise at least a binding (α) chain that binds GM-CSF with low affinity (Gearing *et al.*, 1989) and a second (β) chain that does not seem to bind GM-CSF by itself but which allows the formation of a high affinity receptor when co-expressed with the α -chain (Hayashida *et al.*, 1990). The functions mediated by each chain of the GM-CSF receptor are not yet known.

Despite the multiple *in vitro* and *in vivo* studies with GM-CSF, little is known about regions of the molecule essential for activity and in particular whether different regions participate in binding to high and low affinity receptors and their relationship to function. Using a chemical-synthesis approach we have previously shown that the 14 most N-terminal and the six most C-terminal residues of GM-CSF are not required for function, and importantly that the 14–24 region in the first predicted α -helix of GM-CSF is essential for bioactivity (Clark-Lewis *et al.*, 1988). Similar results were obtained using human–mouse GM-CSF chimeric molecules (Kaushansky *et al.*, 1989; Shanafelt *et al.*, 1991) and monoclonal anti-GM-CSF antibodies which blocked activity (Brown *et al.*, 1990). The latter two approaches identified, in addition, a second region in GM-CSF between residues 88–96 important for activity.

We have now used site-directed mutagenesis to study in more detail the predicted first α -helix of GM-CSF and, in particular, focussed on the hydrophilic residues Gln at position 20 and Glu at position 21. Our results show that residue Glu21 is critical for the full biological activity of GM-CSF. Significantly, substitution of Glu21 with Arg impaired the binding to high affinity but not to low affinity GM-CSF receptors, thus linking high affinity binding to the various functions examined and suggesting that Glu21 may be involved in binding to the β -subunit of the GM-CSF receptor.

Results

Mutagenesis of Gln20 and Glu21 of human GM-CSF

Initial experiments designed to examine the effect of N-terminal deletions on GM-CSF activity revealed that deletion of residues 1–24, 7–24 and 14–24 caused loss of GM-CSF activity (data not shown). We then focused on residues

Gln20 and Glu21 predicted to constitute a hydrophilic face of the GM-CSF molecule (Parry *et al.*, 1991). Deletion of residues 20–21 resulted in the complete loss of GM-CSF ability to stimulate bone marrow colony formation, the neutrophil respiratory burst and antibody-dependent cytotoxicity (ADCC), eosinophil-mediated antibody-dependent cytotoxicity (ADCC), and in the ability to compete for [125 I]GM-CSF high affinity binding to human neutrophils. In order to ascertain the importance of positions 20 and 21 for the biological activities of GM-CSF less severe modifications were carried out by introducing the non-polar residue alanine at both these positions. Experiments using transiently transfected COS cells showed that GM-CSF(Ala20) had the same potency as wild-type (WT) GM-CSF at stimulating day 14 GM bone marrow colonies (100.3% potency, not different to WT GM-CSF $P = 0.9$, $n = 6$), neutrophil ADCC (116.9%, $n = 6$, $P = 0.45$) and superoxide anion (O_2^-) generation (104.3%, $n = 12$, $P = 0.65$) and eosinophil ADCC (94.7%, $n = 4$, $P = 0.76$). In contrast GM-CSF(Ala21) was less potent than WT GM-CSF at stimulating neutrophil ADCC (24.2%, $n = 8$, $P < 0.001$) and O_2^- generation (28.9%, $n = 12$, $P < 0.001$), and eosinophil ADCC (21.0%, $n = 4$, $P < 0.001$). The double substitution GM-CSF(Ala20 Ala21) was also less potent than WT GM-CSF at stimulating neutrophil ADCC (17.0%, $n = 6$, $P < 0.01$) and O_2^- generation (7.5%, $n = 11$, $P < 0.001$). In addition, GM-CSF(Ala21) and GM-CSF(Ala20 Ala21) failed to stimulate 50% of WT GM-CSF day 14 GM colonies at the highest concentrations tested (100 ng/ml).

Single amino acid substitutions of Glu21

Having identified residue 21 as important for several biological properties of GM-CSF, a series of single substitutions were carried out to replace Glu21 with amino acids of different hydrophilicity and polarity. The amino acids introduced were aspartic acid (hydrophilic, acidic), glutamine (hydrophobic, neutral), serine (hydrophilic, neutral), alanine (hydrophobic, neutral), arginine (hydrophilic, basic) and lysine (hydrophilic, basic).

Examination of these GM-CSF mutants for their ability to stimulate the proliferation of leukaemic cells showed that the substitution of Glu for Asp reduced activity by 4-fold, for Ser by 30-fold, for Ala by 80-fold and for Gln by 100-fold. Substitutions with Lys and Arg yielded GM-CSF mutants with no activity up to a concentration of 10 ng/ml (Figure 1A). A titration of these GM-CSF mutants on stimulation of monocyte adherence showed similar results, with the acidic residue Asp affecting GM-CSF activity the least and the basic residues Arg and Lys virtually abolishing GM-CSF activity up to a concentration of 10 ng/ml (Figure 1B). Essentially the same pattern was observed in the stimulation of neutrophil O_2^- generation (data not shown).

Competition binding experiments on neutrophil high affinity GM-CSF receptors mirrored the hierarchy of the biological data. Using 70 pM [125 I]GM-CSF and the different GM-CSF proteins at 30-fold excess the levels of competition were 87.9% for WT GM-CSF, 26.1% for Asp21, 23.0% for Ser21, 18.3% for Ala21, 16.6% for Gln21, 14.6% for Arg21 and 7.4% for Lys21.

Purification and biological activities of GM-CSF with substitutions at positions 20 and 21

To study in more detail the relevance of positions 20 and 21, GM-CSF(Ala20), GM-CSF(Ala21), GM-CSF(Ala20, Ala21), GM-CSF(Arg21) and GM-CSF(Ala20, Arg21) were purified by affinity chromatography and reversed phase HPLC from supernatants of transfected CHO cells. After quantitation by amino acid analysis the GM-CSF mutants were visualized by Western blot analysis (Figure 2A) and silver staining (Figure 2B) to confirm purity and integrity. The purified GM-CSF mutants showed similar molecular weight heterogeneity, hence degree of glycosylation, to the wild-type GM-CSF (Figure 2A and B).

These purified mutants stimulated the [3 H]thymidine incorporation of leukaemic cells with different potencies. While GM-CSF(Ala20) was equipotent to the wild-type GM-CSF, GM-CSF(Ala21) was 30-fold, GM-CSF(Ala20, Ala21) 100-fold and GM-CSF(Arg21) and GM-CSF(Ala20,

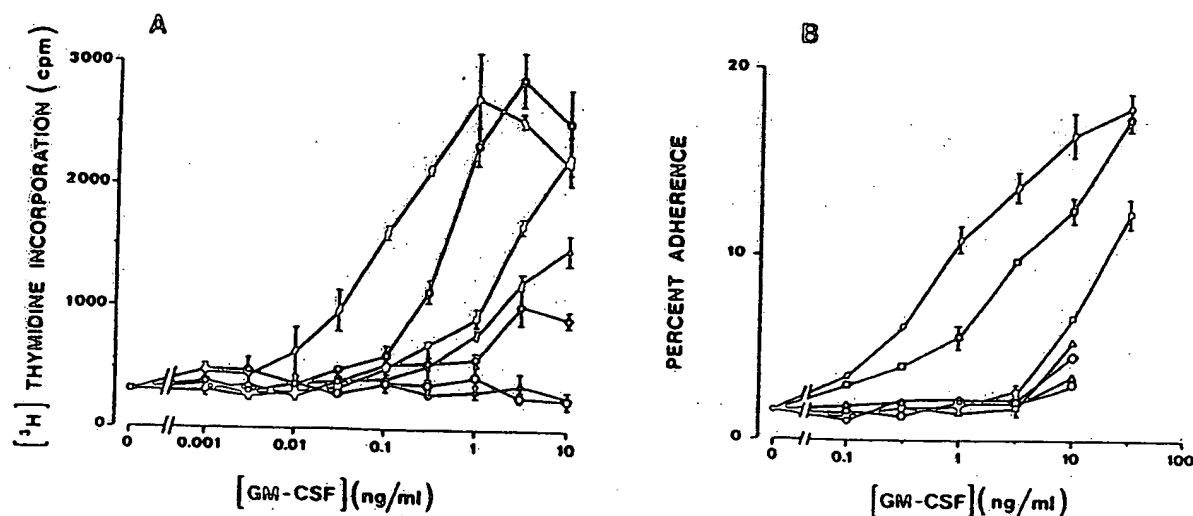


Fig. 1. Titration of GM-CSF mutants containing different residues at position 21 for their ability to stimulate the incorporation of [3 H]thymidine into chronic myeloid leukaemia cells (A) and to stimulate monocyte adherence (B). Wild-type (WT) GM-CSF (\circ) as well as GM-CSF(Ala21) (Δ), GM-CSF(Arg21) (\square), GM-CSF(Lys21) (\circ), GM-CSF(Gln21) (\diamond), GM-CSF(Ser21) (\square) and GM-CSF(Asp21) (\square) were tested at the concentrations shown. Representative experiments are shown with the bars spanning the SEM.

Arg21) 200-fold less potent than wild-type GM-CSF respectively (Figure 3A).

Stimulation of mature cell function showed a similar hierarchy and relative potencies in the stimulation of monocyte adherence and neutrophil O_2^- production. On monocyte adherence GM-CSF(Ala21) was 30-fold less potent than wild-type GM-CSF and GM-CSF(Ala20), while GM-CSF(Ala20, Ala21), GM-CSF(Arg21) and GM-CSF(Ala20, Arg21) were ~100-, 200- and 200-fold less potent respectively (Figure 3B). On neutrophil O_2^-

production GM-CSF(Ala20), GM-CSF(Ala21), GM-CSF(Ala20, Ala21), GM-CSF(Arg21), GM-CSF(Ala20, Arg21) were 10-, 100-, 200- and 200-fold less potent than wild-type GM-CSF respectively (Figure 4A).

In competition binding experiments on the neutrophil high affinity GM-CSF receptor, GM-CSF(Ala20) was 3-fold, GM-CSF(Ala21) 40-fold, and GM-CSF(Ala20, Ala21), GM-CSF(Arg21) and GM-CSF(Ala20, Arg21) > 100-fold less effective than wild-type GM-CSF at competing for [^{125}I]GM-CSF binding (Figure 4B).

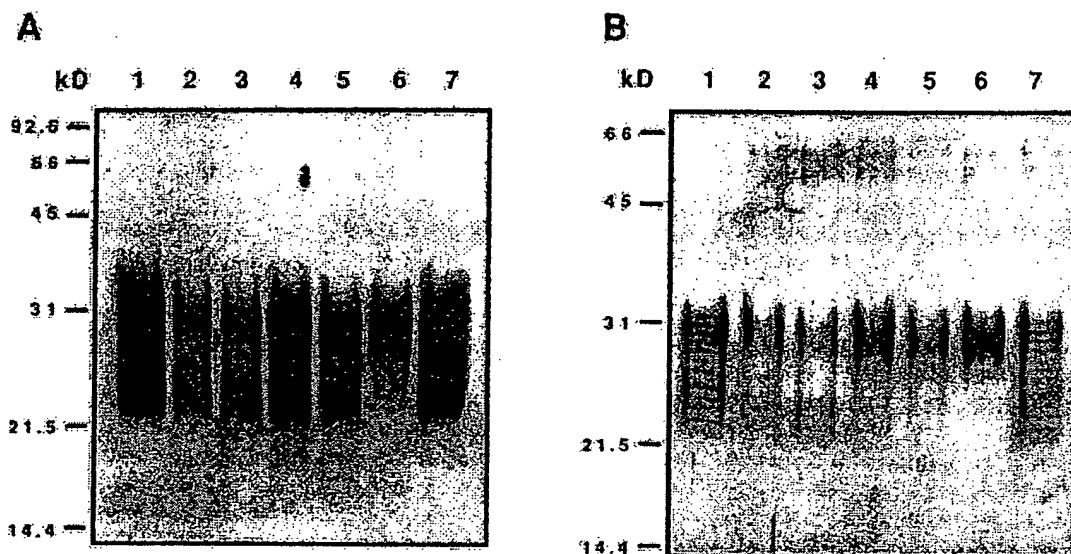


Fig. 2. Western blot analysis (A) and silver staining (B) of GM-CSF mutants after purification by affinity chromatography and HPLC. After quantitation by amino acid analysis 200 ng of protein was loaded per track and electrophoresed on a 12.5% polyacrylamide-SDS gel. In (A) GM-CSF protein was visualized by using a sheep antibody to human GM-CSF followed by a biotinylated rabbit anti-sheep antibody and developing the reaction with diaminobenzidine. In (B) the same amount of purified GM-CSF protein was visualized by silver staining. The different lanes contain WT GM-CSF (lane 1), GM-CSF(Arg21) (lane 2), GM-CSF(Ala20, Arg21) (lane 3), GM-CSF(Ala20) (lane 4), GM-CSF(Ala21) (lane 5), GM-CSF(Ala20, Ala21) (lane 6) and WT GM-CSF (lane 7).

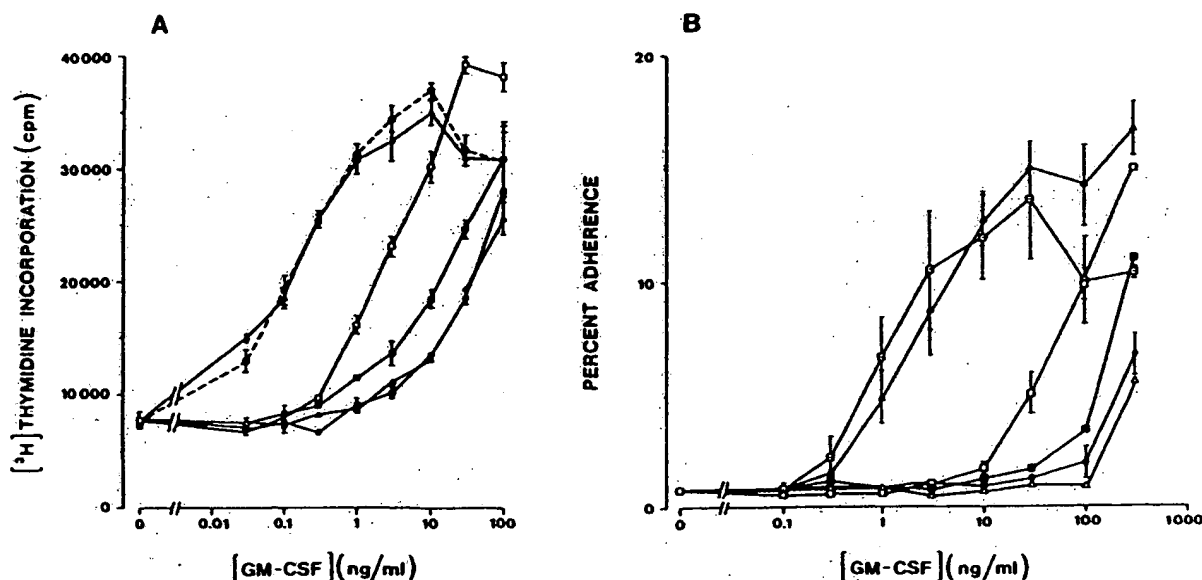


Fig. 3. Titration of purified GM-CSF proteins, WT GM-CSF (\square), GM-CSF(Ala20) (\blacktriangle), GM-CSF(Ala21) (\square), GM-CSF(Arg21) (\bullet), GM-CSF(Ala20, Ala21) (\blacksquare) and GM-CSF(Ala20, Arg21) (\triangle), for their ability to stimulate the [3H]thymidine incorporation in chronic myeloid leukaemic cells (A) and to stimulate monocyte adherence (B). Representative experiments are shown with the bars spanning the SEM.

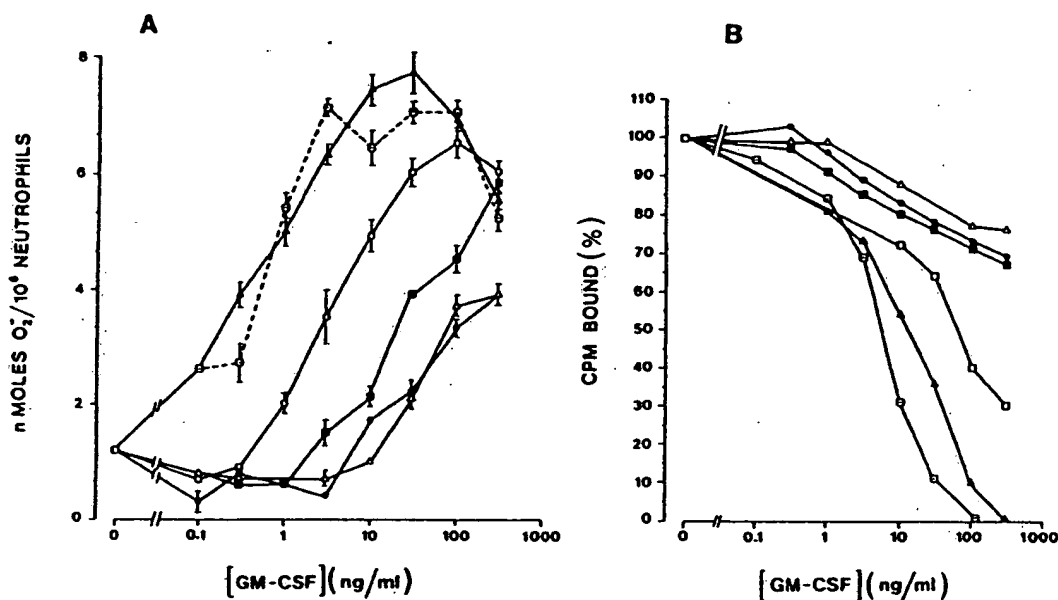


Fig. 4. Comparison of purified GM-CSF mutants for their ability to stimulate neutrophil superoxide production (A), and to compete for the binding of yeast-derived 125 I-labelled GM-CSF to the high affinity GM-CSF receptors of neutrophils (B). The GM-CSF mutants used were as described for Figure 3. Representative experiments are shown with the bars spanning the SEM.

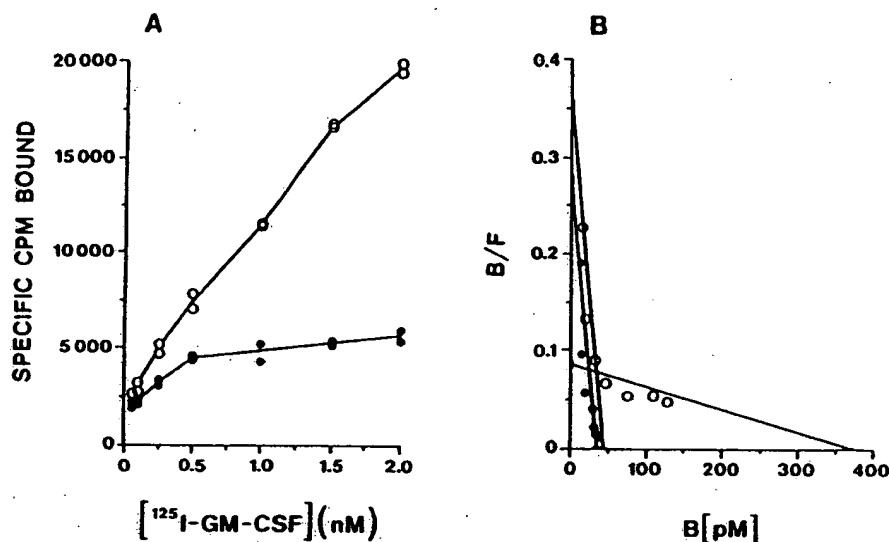


Fig. 5. Competition of yeast-derived [125 I]GM-CSF binding to monocytes by GM-CSF(Arg21). (A) Binding curve with different concentrations of [125 I]GM-CSF in the presence of 100-fold excess GM-CSF (O) or 35-fold excess purified GM-CSF(Arg21) (●). Specific counts for each duplicate determination are shown. (B) Scatchard analyses of the experiment in (A) showing the competition in the presence of GM-CSF (O) or GM-CSF(Arg21) (●). The mean values are plotted. Incubation was for 16 h at 4°C.

Differential binding of GM-CSF(Arg21) to high and low affinity GM-CSF receptors

Although the experiments described above showed that Glu21 was important for several biological activities of GM-CSF and for high affinity binding, they did not distinguish whether binding to the low affinity receptor was also affected and as such could not discriminate which type of receptor was responsible for function. To address this question we selected the GM-CSF(Arg21) mutant which had been shown to be one of the weakest GM-CSF analogues. Purified GM-CSF(Arg21) was tested for its ability to inhibit GM-CSF binding to high and low affinity receptors by using human monocytes which express both types of receptor (Elliott

et al., 1989). A binding curve using increasing concentrations of [125 I]GM-CSF was performed in the presence of 35-fold excess purified GM-CSF(Arg21). This experiment revealed that GM-CSF(Arg21) slightly inhibited the binding of low concentrations of [125 I]GM-CSF (high affinity binding) and strongly inhibited the binding of high concentrations of [125 I]GM-CSF (low affinity binding) (Figure 5A). The percentage levels of inhibition by GM-CSF(Arg21) for each concentration of [125 I]GM-CSF were 13.7% (50 pM), 26.2% (100 pM), 35.6% (250 pM), 37.2% (500 pM), 58.4% (1 nM), 68.7% (1.5 nM) and 71.2% (2 nM). Scatchard transformation of these data showed that GM-CSF(Arg21) eliminated GM-CSF low affinity binding

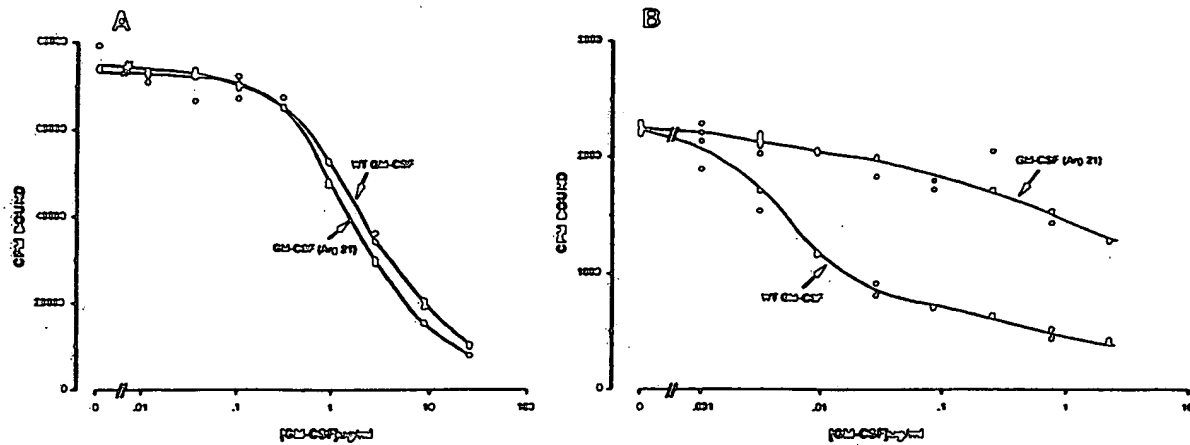


Fig. 6. Competition of *E.coli*-derived [125 I]GM-CSF binding to (A) COS cells transfected with the low affinity α -chain of the human GM-CSF receptor, and to (B) purified human neutrophils by increasing concentrations of (O) WT GM-CSF or (□) GM-CSF(Arg21). Binding was for 3 h at 4°C to (A) 1×10^6 transfected COS cells with 6 nM [125 I]GM-CSF or (B) 1.5×10^6 neutrophils with 2 nM [125 I]GM-CSF.

while slightly decreasing the number of high affinity sites (Figure 5B).

To confirm the ability of GM-CSF(Arg21) to distinguish between high and low affinity receptors experiments were carried out using COS cells transfected with the GM-CSF low affinity α -chain of the GM-CSF receptor (Gearing *et al.*, 1989) and neutrophils as controls. The results showed that the binding of GM-CSF(Arg21) to low affinity GM-CSF receptors was indistinguishable from that of wild-type GM-CSF (Figure 6A) and that GM-CSF(Arg21) was 300-fold less potent than wild-type GM-CSF at competing for neutrophil high affinity receptors (Figure 6B).

Discussion

We have performed a structure–function analysis of human GM-CSF by using site-directed mutagenesis and multiple biological and binding assays. Our results show that residue 21 of GM-CSF is important for GM-CSF function including proliferation, differentiation and mature cell function. We also report that residue 21 is implicated in binding to high affinity as opposed to low affinity GM-CSF receptors thus linking high affinity binding with the multiple functions examined. Significantly, substitution of Glu21 with Arg greatly decreased binding to high affinity receptors but retained the capacity to bind to low affinity receptors strongly suggesting that residue 21 is involved in binding to the β -chain of the GM-CSF receptor.

Residue Gln20 appeared not to be critical in that GM-CSF(Ala20) exhibited the same biological activity as the native GM-CSF. This is in agreement with other experiments in which a mouse–human hybrid GM-CSF molecule containing a mouse sequence between amino acids 1–20 was found to be fully active (Kaushansky *et al.*, 1989; Shanafelt *et al.*, 1991), thus showing that a change from Gln20 to the mouse equivalent Lys20 does not alter GM-CSF activity. However, the finding that GM-CSF(Ala20) had a slightly impaired capacity to compete for high affinity binding (Figure 4B) and the fact that GM-CSF(Ala20, Ala21) was less active than either GM-CSF(Ala20) or GM-CSF(Ala21) suggest some contribution by Gln20 to the activities of GM-CSF.

In contrast to Gln20, substitution of Glu21 by amino acids

of different hydrophilicity and polarity significantly affected the potency of GM-CSF. Biological analysis of these mutants showed that the substitution of an acidic (Glu) for another acidic (Asp) residue caused the least change of activity while substitution by basic residues (Arg, Lys) caused the biggest reduction in GM-CSF activities. The other substitutions, either hydrophilic uncharged or hydrophobic, exhibited intermediate potency.

These series of substitutions suggest that Glu at position 21 is critical for GM-CSF activity and its acidic nature is important for binding to the high affinity GM-CSF receptor or in maintaining an appropriate tertiary configuration. In view of these results it is tempting to speculate that Glu21 is part of a GM-CSF binding site or close to residues involved in it, a notion supported by predictive conformational studies suggesting that Glu21 lies in the external face of the first α -helix (Parry *et al.*, 1991). However, despite the fact that single point mutations can be more revealing than antibody molecules (which are six times the size of GM-CSF) or deletion mutants, the possibility cannot be ruled out that the mutations we made have induced a conformational change in GM-CSF. Nevertheless the fact that GM-CSF(Arg21) was equipotent to WT GM-CSF in competing for low affinity binding would argue against the latter possibility.

An important finding with these GM-CSF mutants was the demonstration that all the activities of GM-CSF tested were affected. Examination of GM-CSF mutant-mediated stimulation of cell proliferation showed the same hierarchy as for stimulation of neutrophil and monocyte function. In competition binding experiments the order in which these mutants inhibited the binding of [125 I]GM-CSF to the high affinity receptor of neutrophils paralleled their bioactivity.

Whilst these studies focused on the 14–24 region of GM-CSF there seem to be other regions in the GM-CSF molecule also important for activity. The fact that a chemically synthesized GM-CSF peptide spanning residues 1–53 was not sufficient for activity (Clark-Lewis *et al.*, 1988) suggests a second region of importance in the C-terminus. This region appears to extend between residues 77 and 94 as shown with human–mouse GM-CSF chimeric molecules (Kaushansky *et al.*, 1989; Shanafelt *et al.*, 1991), and inhibitory monoclonal antibodies that recognize this area (Brown *et al.*, 1990; Nice *et al.*, 1990). In addition the involvement of regions

40–77 and 110–127 has also been suggested based on inhibitory studies using monoclonal antibodies (Nice *et al.*, 1990).

Although mainly two regions of GM-CSF, encompassing residues 14–24 and 77–94, appear to be involved in binding and bioactivity they both need to be preserved for full activity. Neither the peptides 1–53 (Clark-Lewis *et al.*, 1988; Gamble *et al.*, 1990), 54–127 (Clark-Lewis *et al.*, 1988; Gamble *et al.*, 1990) nor 86–93 (Nice *et al.*, 1990) expressed agonistic or antagonistic activity when used separately. These results suggest that either both regions are adjacent in the tertiary structure, forming a single binding site, or that they bind separately to distinct binding sites of the GM-CSF receptor. Our results with GM-CSF(Arg21) showing impairment of binding to high but not low affinity receptors (Figures 5 and 6) argues for the presence of two binding sites and suggests that Glu21 is involved in binding to the β -chain (or a third member of the GM-CSF–receptor complex) but not to the α -chain (Figure 7).

We note that GM-CSF belongs to a family of related cytokines with similar predicted tertiary configuration (Parry *et al.*, 1988) and whose receptors also belong to a common family (Gearing *et al.*, 1989; Bazan 1990). Our model with GM-CSF (Figure 7) may apply to other molecule members of this family, in particular IL-3 and IL-5, both of which have a glutamic acid at positions 22 and 10 respectively which are predicted to lie in the external face of the first α -helix (Parry *et al.*, 1991). Appropriate changes in this region may alter the binding and function of these molecules in a similar way, particularly as the β -chain of the GM-CSF receptor appears to be part of the high affinity IL-3 and IL-5 receptors (Lopez *et al.*, 1990, 1991; Kitamura *et al.*, 1991b; Tavernier *et al.*, 1991).

An important implication of the differential binding to high

and low affinity GM-CSF receptors by GM-CSF(Arg21) is that such a mutant, despite having greatly reduced potency at stimulating high affinity receptor-mediated functions, would be expected to fully occupy and activate low affinity receptor-mediated functions. The low affinity receptor may mediate proliferation signals in some cases as suggested by the relatively high concentrations of GM-CSF required for stimulation of [3 H]thymidine incorporation in monocytes (Elliott *et al.*, 1989) and in particular by the ability of nM but not pM concentrations of human GM-CSF to stimulate the proliferation of mouse FDCP1 cells transfected with the low affinity human GM-CSF receptor α -chain (Metcalf *et al.*, 1990). However, in a similar system but which used instead transfected CTLL cells, the α -chain alone was insufficient to allow proliferation (Kitamura *et al.*, 1991a) and evidence has been presented that interaction with the β -chain across species is required for signalling even though high affinity binding is not observed.

The latter experiment emphasizes a potential therapeutic use for molecules similar to GM-CSF(Arg21), namely their capacity to act as antagonists in situations where the low affinity α -chain provides binding but is incapable of generating cellular signals. Since binding to the α -chain as well as the β -chain is required for high affinity binding and signalling, GM-CSF(Arg21) may pave the way for the engineering of mutants that have totally lost their ability to interact with the high affinity receptor but which by retaining their ability to bind to the α -chain can antagonize the effect of native GM-CSF. Such antagonists, either as monomers or dimers, could have useful clinical applications in situations where the presence of GM-CSF can exacerbate inflammation (Koyanagi *et al.*, 1988) or lead to tumour cell growth (Baldwin *et al.*, 1989; Dedhar *et al.*, 1988; Berdel *et al.*, 1989).

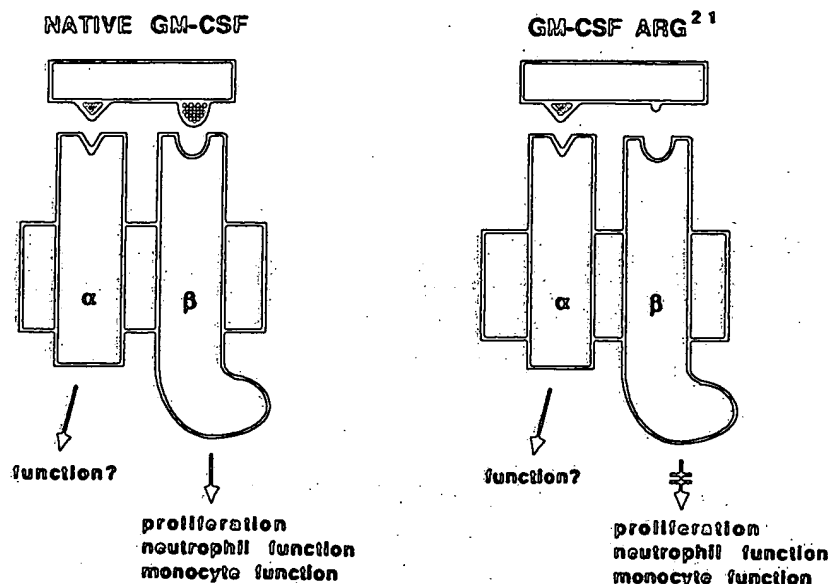


Fig. 7. Proposed model of GM-CSF interaction with the α - and β -chains of the GM-CSF receptor to accommodate the biological and binding data. On the left, binding to both the α - and β -chain of the GM-CSF receptor is shown. The triangular symbol in GM-CSF signifies a distinct structure (probably contributed to by helix D and the loop joining helices C and D) binding the α -chain, and the semi-circular structure, the critical contribution of Glu21 to the β -chain binding. The complex gives high affinity binding and full function. On the right, GM-CSF(Arg21) mutant is shown with an intact interaction with the α -chain (thus giving low affinity binding) but impaired interaction with the β -chain. The possibility that the α -chain by itself is able to stimulate some function in some cells is also indicated on this diagram.

Materials and methods

Site-directed mutagenesis and expression vector constructs

Site-directed mutagenesis of a human GM-CSF cDNA clone kindly provided by Dr S. Clark (Genetics Institute, Cambridge, MA), was performed in plasmid M13 as described (Zoller and Smith, 1984) with mutant plaques being screened by the 3 M tetramethyl ammonium chloride procedure (Wood *et al.*, 1985). The presence of the correct mutation was confirmed by chain termination sequencing (Sanger *et al.*, 1977) using a Sequenase Kit (United States Biochemical, Cleveland, OH). The mutant GM-CSF cDNAs were then excised from the M13 clones RF DNA and subcloned into the transient mammalian expression vector pJL4 (Gough *et al.*, 1985). Several mutant GM-CSF cDNAs were subsequently subcloned from the pJGM constructs into the neomycin selectable, mammalian expression vector pRSVN.07 (gift from Dr A. Robbins, Department of Biochemistry, University of Adelaide). All plasmid constructs were sequenced at the site of the mutation prior to transfection (Chen and Seeburg, 1985).

Transfection of GM-CSF and its analogues

Transient transfections in COS cells. COS cells were grown to 50–70% confluence in Dulbecco's Modified Eagle's medium (DMEM) containing 20 mM HEPES, penicillin, gentamicin and supplemented with 10% fetal calf serum (FCS). DNA constructs were introduced into COS cells by electroporation using a Bio-Rad Gene Pulser (Chu *et al.*, 1987). For each transfection 20 µg of DNA, 25 µg sonicated salmon sperm DNA and 50 µl FCS were mixed with 5×10^6 COS cells in 0.5 ml of 20 mM HEPES-buffered saline containing 6 mM glucose. After a 24 h incubation the medium was replaced with FCS-free DMEM and incubated for a further 72 h before the conditioned medium was harvested and assayed for GM-CSF protein.

Permanent transfections in CHO cells. CHO cells were grown to ~80% confluence in Hams F12 nutrient mixture containing penicillin, gentamicin and supplemented with 10% FCS. DNA constructs were introduced into CHO cells by electroporation after mixing 10 µg of pRSVNGM plasmid DNA with 5×10^6 CHO cells. 24–48 h after transfection selective F12 media containing geneticin (Gibco Laboratories) was added to the cells. Maximally expressing individual geneticin-resistant CHO colonies were selected and used to produce GM-CSF or mutant analogues.

Visualization of mutant GM-CSF protein

GM-CSF-containing COS cell supernatants and purified GM-CSF protein was size-fractionated by SDS–12.5% PAGE (Laemmli, 1970). For Western blot analysis, protein was transferred to nitrocellulose as described (Towbin *et al.*, 1979). Filters were probed with a sheep anti-GM-CSF (gift from Dr S. Clark, Genetics Institute, Cambridge, MA) followed by a second layer of biotinylated rabbit anti-sheep IgG. After a further incubation with an avidin-biotinylated horseradish peroxidase conjugate, the complex was visualized using a diaminobenzidine substrate solution. For silver staining, the method of Morrissey (1981) was used.

Quantitation and purification of GM-CSF protein

The amount of GM-CSF protein present in COS cell supernatants was quantitated by a radioimmunoassay (RIA). Some mutants were selected for purification and quantitation by amino acid analysis.

RIA. A competitive RIA was developed using 125 I-labelled human GM-CSF and a polyclonal sheep anti-GM-CSF serum (gift from Dr S. Clark, Genetics Institute, Cambridge, MA). GM-CSF modified by the addition of an extra tyrosine in the N-terminus (gift from Dr L.S. Park, Immunex Corp., Seattle, WA) was labelled as described below. COS cell supernatants (50 µl) were incubated with sheep anti-GM-CSF serum (50 µl of 1:40,000 dilution). After 4 h incubation at 4°C, 0.1 ng of [125 I]GM-CSF was added for a further 16 h before adding 100 µl of reconstituted anti-sheep Immunobead reagent (Bio-Rad Laboratories, Richmond, CA) for 4 h. The mixtures were then washed twice with phosphate-buffered saline (PBS), the pellet resuspended in 200 µl of PBS and transferred to 3DT tubes for counting in a gamma-counter (Packard Instrument Company, Meriden, CT). The amount of GM-CSF protein was calculated from a standard curve constructed with known amounts of GM-CSF.

Protein purification and amino acid analysis. GM-CSF protein in the supernatants of CHO cell lines permanently transfected with selected GM-CSF mutant cDNA was purified using an affinity column containing the monoclonal antibody LMM111 attached to Sepharose beads (Cebon *et al.*, 1988). Further purification was achieved by reversed phase HPLC and the resulting GM-CSF protein quantitated by amino acid analysis as described (Cebon *et al.*, 1990). This procedure also calculated the GM-CSF mutant preparations to be >99% pure.

Stimulation of haemopoietic cell proliferation

Two types of assay were performed.

Colony assay. This assay measured the clonal proliferation and differentiation of bone marrow progenitor cells in semi-solid agar and was carried out as described (Lopez *et al.*, 1988).

Proliferation of chronic myeloid leukaemic (CML) cells. Primary CML cells from one patient were selected for their ability to incorporate [3 H]thymidine in response to GM-CSF. This assay was performed as described (Lopez *et al.*, 1988).

Functional activation of human granulocytes and monocytes

Antibody-dependent cell-mediated cytotoxicity (ADCC) assay. Neutrophils and eosinophils were purified on Metrizamide (Nyegaard, Oslo) and tested against antibody-coated, 51 Cr-labelled P815 cells coupled with trinitrophenyl as previously described (Vadas *et al.*, 1983).

Superoxide anion production assay. This was carried out as described previously (Lopez *et al.*, 1986).

Monocyte adherence. Monocytes were purified from the peripheral blood of normal donors obtained from the Adelaide Red Cross Transfusion Service, as previously described (Elliott *et al.*, 1990). Stimulation of monocyte adhesion by GM-CSF was measured by an isotopic method essentially as described (Elliott *et al.*, 1990). In each assay the concentration of GM-CSF protein giving 50% maximal response was determined and the relative potency calculated by dividing the concentration of WT GM-CSF giving 50% stimulation by the concentration of mutant GM-CSF protein giving 50% stimulation and multiplying by 100. In some experiments the geometric mean from several experiments was calculated and statistical significance between values from different mutants determined by a paired *t* test.

Radio-receptor assay

Radioiodination of GM-CSF. Yeast-derived human GM-CSF (gift from Dr L. Park, Immunex Corporation, Seattle, WA), or *Escherichia coli*-derived human GM-CSF was radioiodinated by the ICI method (Contreras *et al.*, 1983). Iodinated protein was separated from free 125 I by chromatography on a Sephadex G-25 PD 10 column (Pharmacia, Uppsala, Sweden) equilibrated in PBS containing 0.02% Tween 20, and stored at 4°C for up to 4 weeks. Before use, the iodinated protein was purified from Tween and non-protein-associated radioactivity by cation exchange chromatography on a 0.3 ml CM-Sephacrose CL-6B column (Pharmacia) and stored at 4°C for up to 5 days. The radiolabelled GM-CSF retained >90% biological activity as judged from titration curves using non-iodinated GM-CSF as controls.

Competition binding assays. Competition for binding to high affinity receptors used freshly purified neutrophils which express only this type of receptor (Gasson *et al.*, 1986). The cells were suspended in binding medium consisting of RPMI 1640 supplemented with 20 mmol/l HEPES, 0.5% bovine serum albumin (BSA) and 0.1% sodium azide. Typically, equal volumes (50 µl) of 4×10^6 neutrophils, 70 pM iodinated GM-CSF, and different concentrations of GM-CSF and GM-CSF analogues were mixed in siliconized glass tubes for 3 h at 4°C. Competition for binding to high and low affinity GM-CSF receptors used human monocytes which express both types of receptor (Elliott *et al.*, 1989). Equal volumes (50 µl) of cells (2×10^6), iodinated GM-CSF at different concentrations and 100-fold excess wild-type GM-CSF to establish non-specific binding, or 35-fold excess GM-CSF(Arg21) were mixed together for 16 h at 4°C before centrifugation of the cells over a cushion of FCS as above. Specific counts were determined by first subtracting the counts obtained in the presence of excess wild-type GM-CSF. Competition for binding to the low affinity GM-CSF receptor was performed using COS cells transiently transfected with a GM-CSF receptor cDNA clone as described (Gearing *et al.*, 1989). To 10^6 COS cells 6 nM of *E. coli*-derived [125 I]GM-CSF were added in the presence of different concentrations of wild-type GM-CSF or GM-CSF(Arg21) for 3 h at 4°C. In each case cell suspensions were overlaid on 0.2 ml FCS at 4°C, centrifuged in a Beckman Microfuge 12, and the tip of each tube containing the visible cell pellet cut off and counted in a gamma counter. In the case of monocytes the results are expressed in the form of equilibrium binding data and Scatchard transformation of these data as described (Scatchard, 1949).

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References

- Antman, K.S., Griffin, J.D., Elias, A., Socinski, M.A., Ryan, L., Cannistra, S.A., Oette, D., Whitley, M., Frei, E. and Schnipper, L.E. (1988) *N. Engl. J. Med.*, **319**, 593–598.
- Baldwin, G.C., Gasson, J.C., Quan, S.G., Fleischmann, J., Weisbart, R., Oette, D., Mitsuyasu, R.T. and Golde, D.W. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 2763–2766.
- Baldwin, G.C., Gasson, J.C., Kaufman, S.E., Quan, S.G., Williams, R.E., Avalos, B.R., Gaxdar, A.F., Golde, D.W. and DiPersio, J.F. (1989) *Blood*, **73**, 1033–1037.
- Bazan, J.F. (1990) *Immunol. Today*, **11**, 350–354.
- Berdel, W.E., Danhauser-Reidl, S., Steinhäuser, G. and Winton, E.F. (1989) *Blood*, **73**, 80–83.
- Brown, C.B., Hart, C.E., Curtis, D.M., Bailey, M.C. and Kaushansky, K. (1990) *J. Immunol.*, **144**, 2184–2189.
- Cebon, J., Dempsey, P., Fox, R., Kannourakis, G., Bonnem, E., Burgess, A.W. and Morstyn, G. (1988) *Blood*, **72**, 1340–1347.
- Cebon, J., Nicola, N., Ward, M., Gardner, I., Dempsey, P., Layton, J., Dührsen, U., Burgess, A., Nice, E. and Morstyn, G. (1990) *J. Biol. Chem.*, **265**, 4483–4489.
- Chen, E.Y. and Seeburg, P.H. (1985) *DNA*, **4**, 165–170.
- Chu, G., Hayakawa, H. and Berg, P. (1987) *Nucleic Acids Res.*, **15**, 1311–1326.
- Clark-Lewis, I., Lopez, A.F., To, L.B., Vadas, M.A., Schrader, J.W., Hood, L.E. and Kent, S.B.H. (1988) *J. Immunol.*, **141**, 881–889.
- Contreras, M.A., Bale, W.F. and Spar, I.L. (1983) *Methods Enzymol.*, **92**, 277–292.
- Dedhar, S., Gaboury, L., Galloway, P. and Eaves, C. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 9253–9257.
- Elliott, M.J., Vadas, M.A., Eglinton, J.M., Park, L.S., To, L.B., Cleland, L.G., Clark, S.C. and Lopez, A.F. (1989) *Blood*, **74**, 2349–2359.
- Elliott, M.J., Vadas, M.A., Cleland, L.G., Gamble, J.R. and Lopez, A.F. (1990) *J. Immunol.*, **145**, 167–176.
- Gamble, J.R., Elliott, M.J., Jaipargas, E., Lopez, A.F. and Vadas, M.A. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 7169–7173.
- Gamble, J.R., Rand, T.H., Lopez, A.F., Clark-Lewis, I. and Vadas, M.A. (1990) *Exp. Hematol.*, **18**, 897–902.
- Gasson, J.C., Weisbart, R.H., Kaufman, S.E., Clark, S.C., Hewick, R.M., Wong, G.G. and Golde, D.W. (1984) *Science*, **226**, 1339–1342.
- Gasson, J.C., Kaufman, S.E., Weisbart, R.H., Tomonaga, M. and Golde, D.W. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 669–673.
- Gearing, D.P., King, J.A., Gough, N.M. and Nicola, N.A. (1989) *EMBO J.*, **8**, 3667–3676.
- Gough, N.M., Metcalf, D., Gough, J., Grail, D. and Dunn, A.R. (1985) *EMBO J.*, **4**, 645–654.
- Grabstein, K.H., Urdal, D.L., Tushinsky, R.J., Mochizuki, D.Y., Price, V.L., Cantrell, M.A., Gillis, S. and Conlon, P.J. (1986) *Science*, **232**, 506–508.
- Groopman, J.E., Mitsuyasu, R.T., De Leo, M.J., Oette, D.H. and Golde, D.W. (1987) *N. Engl. J. Med.*, **317**, 593–598.
- Hayashida, K., Kitamura, T., Gorman, D.M., Arai, K., Yokota, T. and Miyajima, A. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 9655–9659.
- Kaushansky, K., Shoemaker, S.G., Alfaro, S. and Brown, C. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 1213–1217.
- Kitamura, T., Hayashida, K., Sakamaki, K., Yokota, T., Arai, K. and Miyajima, A. (1991a) *Proc. Natl. Acad. Sci. USA*, **88**, 5082–5086.
- Kitamura, T., Sato, N., Arai, K.-I. and Miyajima, A. (1991b) *Cell*, **66**, 1165–1174.
- Koyanagi, Y., O'Brien, W.A., Zhao, J.Q., Golde, D.W., Gasson, J.C. and Chen, I.S.Y. (1988) *Science*, **241**, 1673–1675.
- Laemmli, U.K. (1970) *Nature*, **227**, 680–685.
- Lopez, A.F., Williamson, D.J., Gamble, J.R., Begley, C.G., Harlan, J.M., Klebanoff, S., Waltersdorff, A., Wong, G., Clark, S. and Vadas, M.A. (1986) *J. Clin. Invest.*, **78**, 1220–1228.
- Lopez, A.F., Dyson, P.G., To, L.B., Elliott, M.J., Milton, S.E., Russell, J.A., Juttner, C.A., Yang, Y.-C., Clark, S.C. and Vadas, M.A. (1988) *Blood*, **72**, 1797–1804.
- Lopez, A.F., Eglinton, J.M., Lyons, A.B., Tapley, P.M., To, L.B., Park, L.S., Clark, S.C. and Vadas, M.A. (1990) *J. Cell. Physiol.*, **145**, 69–77.
- Lopez, A.F., Vadas, M.A., Woodcock, J., Milton, S.E., Lewis, A., Elliott, M.J., Gillis, D., Ireland, R., Otwell, E. and Park, L.S. (1991) *J. Biol. Chem.*, **266**, 24741–24747.
- Metcalf, D., Begley, C.G., Johnson, G.R., Nicola, N.A., Vadas, M.A., Lopez, A.F., Williamson, D.J., Wong, G.G., Clark, S.C. and Wang, E.A. (1986) *Blood*, **67**, 37–45.
- Metcalf, D., Nicola, N.A., Gearing, D.P. and Gough, N.M. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 4670–4674.
- Morrissey, J.H. (1981) *Anal. Biochem.*, **117**, 307–310.
- Nice, E., Dempsey, P., Layton, J., Morstyn, G., Cui, D.F., Simpson, R., Fabri, L. and Burgess, A. (1990) *Growth Factors*, **3**, 159–169.
- Parry, D.A.D., Minasian, E. and Leach, S.J. (1988) *J. Mol. Recogn.*, **1**, 107–110.
- Parry, D.A.D., Minasian, E. and Leach, S.J. (1991) *J. Mol. Recogn.*, **4**, 63–75.
- Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
- Scatchard, G. (1949) *Ann. NY Acad. Sci.*, **51**, 660–672.
- Shanafelt, A.B., Johnson, K.E. and Kastelein, R.A. (1991) *J. Biol. Chem.*, **266**, 13804–13810.
- Sieff, C.A., Emerson, S.G., Donahue, R.E., Nathan, D.G., Wang, E.A., Wong, G.G. and Clark, S.C. (1985) *Science*, **230**, 1171–1173.
- Tavernier, J., Devos, R., Cornelis, S., Tuypens, T., Van der Heyden, J., Fiers, W. and Plactinck, G. (1991) *Cell*, **66**, 1175–1184.
- Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350–4354.
- Vadas, M.A., Nicola, N.A. and Metcalf, D. (1983) *J. Immunol.*, **130**, 795–799.
- Wood, W.I., Gitschier, J., Lasky, L.A. and Lawn, R.M. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 1585–1588.
- Zoller, M.J. and Smith, M. (1984) *DNA*, **3**, 479–488.

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Shanafelt et al. [EMBO J., **10**, 4105–4112 (1991)] have recently shown that the amino terminal helix of GM-CSF governs high affinity binding.

6

Requirement of Hydrophilic Amino-terminal Residues for Granulocyte-Macrophage Colony-stimulating Factor Bioactivity and Receptor Binding*

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a glycoprotein required for the proliferation and differentiation of granulocyte and macrophage precursors. Previous investigations have identified regions in human and murine GM-CSF that are required for bioactivity. In the present study, alanine substitution mutagenesis was undertaken to define more precisely specific amino-terminal residues in murine GM-CSF that are involved in bioactivity and receptor binding. Five double alanine mutants were identified that showed at least 10-fold reductions in bioactivity (K14AK20A, K14AE21A, H15AK20A, H15AE21A, K20AE21A). Each of these mutants maintained a normal *N*-linked glycosylation pattern when expressed in COS-1 cells, suggesting that native polypeptide backbone conformation was preserved. The purified prokaryotic expression products of two mutants (K14AE21A and H15AE21A) had a 100-fold decrease in bioactivity and a decrease in receptor binding, indicating that the side chains of K14, H15, and E21 are required for optimal receptor binding and maximal bioactivity.

Granulocyte-macrophage colony-stimulating factor (GM-CSF)¹ is a glycoprotein required for the proliferation and maturation of cells of the granulocyte and macrophage lineages (1). cDNAs for murine and human GM-CSF have been cloned, and protein was expressed in both prokaryotic and eukaryotic systems (2-5). There is 54% interspecies amino acid identity, yet there is no biologic cross-reactivity. Structure-function studies to date have identified multiple regions throughout murine and human GM-CSF that are required for normal function (5-15). These studies have utilized two basic approaches. In the first, the bioactivity and receptor binding of substitution mutants, deletion mutants, peptides, and interspecies chimeras were assessed. The second approach has been immunologic, utilizing neutralizing monoclonal antibodies

to map critical epitopes. These analyses have identified critical regions which correspond or overlap with predicted α -helices or are located in proposed interhelical loop sequences. While these studies have generated a great deal of information regarding bioactivity and receptor binding, there has been little or no attempt to demonstrate the conformational alterations caused by the various mutations. Therefore, it is not clear if loss in bioactivity results from alteration and/or deletion of specific amino acid side chains or secondary conformational changes.

We have shown previously that *N*-linked glycosylation can be used as an independent measure of conformational changes in mGM-CSF mutants. Both human and murine GM-CSF have two potential *N*-linked glycosylation sites. When mGM-CSF is produced in a mammalian expression system, the predominant species has one *N*-glycosylation site filled (12). In our studies, removal of disulfide bonds or core hydrophobic side chains and disruption of α -helices by proline substitution mutagenesis caused aberrant glycosylation and usually decreased bioactivity when expressed in COS-1 cells (5, 12). In these deletion or substitution mutants, hyperglycosylation was present, with both *N*-linked glycosylation sites utilized. Although not every hyperglycosylation mutant showed significantly decreased bioactivity, all mutants with loss of bioactivity showed abnormal *N*-linked glycosylation. More recent studies comparing the circular dichroism spectra of the proline substitution mutants to GM-CSF show a decreased α -helical content in mutants with decreased bioactivity and hyperglycosylation.² Since mutations which alter the final folded conformation of mGM-CSF are likely to expose the second *N*-linked glycosylation site, the glycosylation pattern of mGM-CSF serves as a sensitive measure of conformational changes induced by mutation.

In the present study, we sought to define further the amino acid residues in the putative amino-terminal α -helix of mGM-CSF that are involved in receptor binding (13, 15). Alanine substitutions were systematically introduced, in an effort to eliminate the contribution of specific side chains to receptor binding, while inducing minimal, if any, alteration of backbone conformation. To assess whether specific alanine substitutions did alter secondary or tertiary structure, mutant proteins were expressed in COS-1 cells, and the degree of glycosylation was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. To confirm that loss of bioactivity was due to reduced receptor binding, selected mutants with normal glycosylation were expressed in *Escherichia coli*,

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¹ The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; mGM-CSF, murine GM-CSF; HPLC, high performance liquid chromatography.

² S. Altmann, L. Melton, M. Prystowsky, and B. Erickson, manuscript in preparation.

purified to homogeneity, and analyzed in both receptor-binding competition studies and bioactivity assays.

The amino-terminal region of murine GM-CSF (mGM-CSF) that includes amino acid residues 11–23 was selected for alanine substitution mutagenesis. This region has been shown to be critical for normal function (6–10, 12–15) and is predicted to have α -helical secondary structure (7, 8, 16). Given that hydrophilic side chains are likely to be exposed to solvent (17, 18) and available to interact with cell-surface receptors, these residues were targeted for mutagenesis.

MATERIALS AND METHODS

Mutagenesis—Site-directed mutagenesis was performed as previously described (12). Native murine GM-CSF cDNA was cloned into pRJB, and plasmid DNA was propagated in the *E. coli* strain XL1-Blue (Stratagene). Site-directed mutagenesis was performed using the Amersham oligonucleotide-directed *in vitro* mutagenesis system (Version 2, RPN.1523). Oligonucleotides 21–22 bases long, complementary to the mGM-CSF cDNA sequence of interest and containing the mutant codon of interest, were utilized. Large scale DNA preparations were made from transformed *E. coli*, and plasmids were twice banded on CsCl gradients for transfection. The entire coding regions of all mutant cDNA were sequenced. Mutants were named as follows: the substituted amino acid(s) position number(s) are preceded by the single initial(s) representing the native residue(s) and followed by the initial(s) of the substituted mutant amino acid(s). For example, the substitution of alanines at positions 14 and 15, for lysine and histidine, respectively, is denoted K14AH15A.

Protein Expression and Bioactivity Quantitation—Transfection was performed using the DEAE-dextran method, as described (12). Ten μ g of plasmid DNA were used for each transfection, with duplicate plates set up to allow for simultaneous production of metabolically radiolabeled and nonlabeled protein products. Briefly, following 24-h incubation of transfected COS-1 cells at 37 °C, the medium was aspirated and replaced with 2.5 ml of methionine-deficient culture medium either with or without 100 μ Ci/ml of [³⁵S]methionine (ICN Biomedicals Trans³⁵S-label, 51006). The supernatants were harvested after 48 h of additional incubation at 37 °C. 1 ml of radiolabeled supernatant was precleared twice with 200 μ l of IgSorb (Enzyme Center, Malden, MA). 200 μ l of precleared supernatant was mixed with 10 μ l of polyclonal rabbit-anti-murine GM-CSF, and the complexes precipitated with IgSorb. Pellets were then resuspended in Laemmli running buffer (19), boiled for 2 min, and the supernatants were electrophoresed on 15% polyacrylamide gels. Prestained high molecular weight markers (BRL) were used as standards. Gels were vacuum-dried, and autoradiography was performed.

16-point 3-fold serial dilution curves from DA3 cell (20) proliferation assays were used to calculate the percent COS-1-conditioned medium for each mutant required for 50% maximal DA3 proliferation (12). In these assays, DNA synthesis by the DA3 cell line was reflected by [³H]thymidine incorporation by cells in 96-well culture plates. The Y value (counts/min) for 50% maximal stimulation was calculated as follows: $0.5 \times (Y_{\text{max}} - Y_{\text{min}})$, where Y_{max} is the average Y value for the upper plateau of the native GM-CSF titration curve, and Y_{min} is the average of all mock transfection values. A least squares algorithm was used to calculate the a and b values for the equation $Y = aX^b$, for the linear portion of the native GM-CSF titration curve. Protein bioactivity for GM-CSF mutants was then calculated by using the least squares algorithm to fit the curve to each of the mutant titration curves and calculate the %COS conditioned medium required for 50% maximal DA3 stimulation. Bioactivity was defined as $100 \times$ the reciprocal of the %COS supernatant at 50% maximal stimulation.

Relative immunoreactive protein content of each transfection supernatant was measured by volume integration of relevant bands on scanned exposed PhosphorImager screens or autoradiograms (Molecular Dynamics) from immunoprecipitation experiments. Counts from the mock transfection were subtracted from all other raw values to derive reported values. The quotient of bioactivity/protein content for each mutant is the specific activity used for comparison to native mGM-CSF. Each experiment was repeated at least twice, and the average values were reported. The total estimated error was approximately 5-fold, including protein quantitation and bioactivity measurements (12).

Prokaryotic Expression—The coding regions of mGM-CSF, K14AE21A, and H15AE21A were subcloned into the pET3A plasmid

and expressed in the bacterial host strain BL21(DE3) pLysS (21). 0.4 mM isopropyl-1-thio- β -D-galactopyranoside was used for induction of T7 RNA polymerase in exponentially growing bacteria. After 3 additional hours of culture at 37 °C, the cells were pelleted and resuspended in lysis buffer A (50 mM Tris-HCl, 2 mM EDTA, 0.1% Triton X, pH 8.0). The suspension was sonicated until smooth and centrifuged. The pellet was resuspended in lysis buffer B (50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, pH 8.0) and sonicated on ice. After centrifugation, the pellet was resuspended in Buffer B containing 1 M guanidine HCl, and sonication was repeated. This step was repeated with Buffer B containing 5 M guanidine HCl. Following centrifugation, the supernatant containing crude protein was harvested for refolding.

Refolding was performed as previously described for IL-4 (22). Crude protein was diluted to a concentration of 0.5 mg/ml in buffer B containing 5 M guanidine HCl, containing 0.2 mM oxidized and 0.2 mM reduced glutathione (Sigma). 9 volumes of the same solution containing only 0.5 M guanidine HCl was added dropwise over 3–4 h at room temperature. The final solution was dialyzed against phosphate-buffered saline for 48 h at 4 °C. Purification of mGM-CSF and mutants was performed using reverse-phase chromatography (Vydac C4 column) with a gradient from 30 to 55% acetonitrile in aqueous trifluoroacetic acid (0.1%, v/v). GM-CSF and mutants eluted as a single peak between 42 and 50% acetonitrile. Collected fractions were lyophilized and resuspended in water. Protein quantification was performed using UV absorbance spectroscopy at 225 nm and 215 nm, with bovine serum albumin standards. DA3 bioactivity assays were performed using 2-fold serial dilutions of purified proteins (12). The protein content required for 50% maximal stimulation of target cells was calculated as described above.

Binding Analysis—Receptor binding analyses were performed using purified prokaryotic expression products. [¹²⁵I]-mGM-CSF (400 Ci/mmol) was radioiodinated using the Bolton-Hunter reagent (Amersham). Competitive binding assays were performed with 2×10^6 acid-washed NFS60 cells using 30 pM [¹²⁵I]-mGM-CSF at 4 °C over a period of 5 h, and results were quantitated using the LIGAND binding program, which permits the resolution of multiple classes of binding sites and dissociation constants (23). Under these conditions, equilibrium is achieved, and both high and low affinity binding sites for mGM-CSF can be detected.³ The F test was used to discriminate between a one- versus a two-site binding model (23). Values of F (and p) for a two-site model giving a better fit of the data than a one-site model were: mGM-CSF, $F = 229$ ($p < 0.00005$); K14AE21A, $F = 0.95$ ($p = 0.42$); and H15AE21A, $F = 0.22$ ($p = 0.81$).

RESULTS

Alanine substitution mutants involving all hydrophilic amino acids from residue 11 to residue 23 were constructed, using an *in vitro* oligonucleotide-primed site-directed mutagenesis kit (Amersham) and expressed in COS-1 cells (12, 13). The relative specific activity of each mutant was calculated by quantitating both bioactivity and immunoreactive protein concentration from COS-conditioned media and comparing the quotient to that of native mGM-CSF.

The relative specific activity of each mutant is listed in Table I. Each of the single alanine mutants retained essentially native bioactivity. However, five mutants with two alanine substitutions had significant reductions in relative specific activity (defined as at least a 10-fold reduction in relative specific activity). These were K14AK20A (13-fold reduction), H15AK20A (28-fold reduction), H15AE21A (160-fold reduction), K20AE21A (160-fold reduction), and K14AE21A (400-fold reduction). As shown in Fig. 1, each of these mutants and native GM-CSF have three major molecular weight species corresponding to 0, 1, or 2 of the potential *N*-linked glycosylation sites being filled. Densitometric analysis of *N*-linked glycosylation species showed similar distributions for each GM-CSF mutant. Within each major species there are several potential *O*-glycosylation forms. While K14AK20A showed an abnormal *O*-linked glycosylation pattern, previous studies indicated that alterations of *O*-gly-

³ A. B. Shanafelt and R. A. Kastelein, unpublished observations.

TABLE I

Mutant relative specific activity

The results of immunoprecipitation and bioactivity assays were used to compare the specific activity of each mutant to that of native mGM-CSF (12). Bioactivity was defined as $100 \times$ the reciprocal of the % COS supernatant that resulted in 50% maximal stimulation of DA3 cells. Relative protein content of each transfection supernatant was measured by volume integration of relevant bands on scanned exposed PhosphorImager screens or autoradiograms (Molecular Dynamics) following immunoprecipitation and electrophoresis. The quotient of bioactivity/protein content for each mutant is the specific activity used for comparison to native mGM-CSF.

Sample	Relative specific activity %
GM-CSF	100
R11A	59
K14A	210
H15A	52
K17A	160
K20A	77
E21A	47
R11AK14A	86
R11AE17A	70
R11AK20A	79
K14AE15A	17
K14AE17A	58
K14AK20A	7.5
K14AE21A	0.25
H15AE17A	38
H15AK20A	33
H15AE21A	0.62
E17AE21A	65
K20AE21A	0.62

ylation in GM-CSF do not impact upon bioactivity (13, 24). Since there is no evidence of significant conformational change in these five mutants as shown by normal *N*-linked glycosylation, it is likely that the decreased bioactivity is related to the loss of critical side chain functionality and interaction with the GM-CSF receptor.

To document that decreased bioactivity in the alanine substitution mutants was a consequence of decreased receptor affinity, competitive binding analyses were performed. Complementary DNAs from native mGM-CSF and mutants with at least 100-fold reductions in bioactivity (K14AE21A, H15AE21A, K20AE21A) were subcloned into the pET3A prokaryotic expression plasmid and expressed in the *E. coli* B strain BL21(DE3)pLysS (21). Protein products were extracted, solubilized, refolded, and purified by reverse-phase HPLC. Homogeneity was documented by silver-staining of polyacrylamide gels and by demonstrating single peaks on analytical reverse-phase HPLC. K20AE21A was expressed poorly in this prokaryotic system and showed an aberrant electrophoretic migration pattern; therefore, binding analyses were performed only on K14AE21A and H15AE21A. Fig. 2 shows bioactivity titration curves for native mGM-CSF, K14AE21A, and H15AE21A. The calculated half-maximal responses for each of these proteins are 160 pg/ml for GM-CSF (relative specific activity = 100%), 21 ng/ml for K14AE21A (relative specific activity = 0.76%), and 23 ng/ml for H15AE21A (relative specific activity = 0.70%).

Competitive binding (Fig. 3) of purified mGM-CSF (versus 125 I-mGM-CSF) exhibited two affinity sites, one of 7.5 nM and the other of 58 pM, reflecting the formation of the low affinity receptor (GM-CSF/GM-CSF receptor α) and the high affinity receptor complex (GM-CSF/GM-CSF receptor α plus β), respectively (23, 25). Both double mutants had only one binding site, K14AE21A having a dissociation constant of 5.0 nM and H15AE21A having a 10-fold lower dissociation constant of 62 nM. The binding affinity of these two double

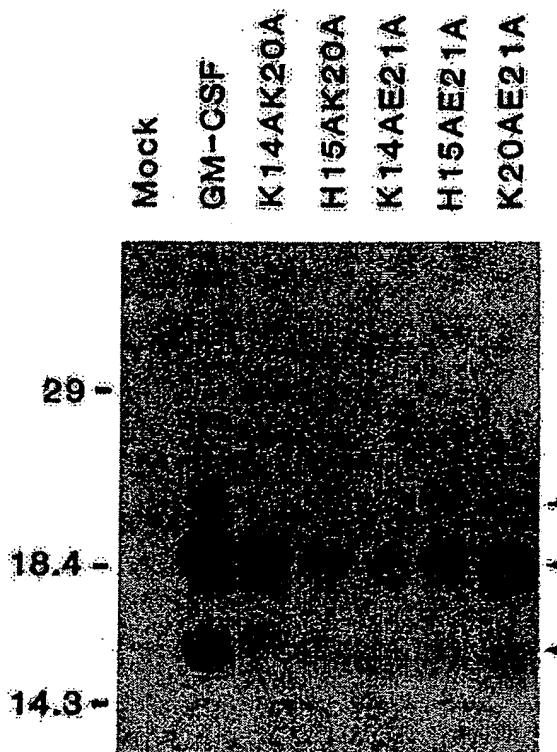


FIG. 1. Mutant electrophoresis. Murine GM-CSF and mutants were expressed in COS-1 cells. Immunoreactive protein was precipitated from metabolically 35 S-labeled COS-1 conditioned media with polyclonal rabbit anti-murine GM-CSF and electrophoresed on a 15% polyacrylamide gel (12). The gel was vacuum-dried, and autoradiography was performed. Molecular weight standards are listed on the left (Bethesda Research Laboratories). Mock, mock transfection; GM-CSF, granulocyte-macrophage colony-stimulating factor; mutants are identified by single initial abbreviations. Arrowheads identify major *N*-linked glycosylation species. The ratio of each *N*-linked glycosylation species for each mutant is (2N:1N:0N): mGM-CSF = 0.42:1.0:0.58, K14AK20A = 0.47:1.0:0.48, H15AK20A = 0.70:1.0:0.31, K14AE21A = 0.41:1.0:0.38, H15AE21A = 0.65:1.0:0.27, K20AE21A = 0.49:1.0:0.41.

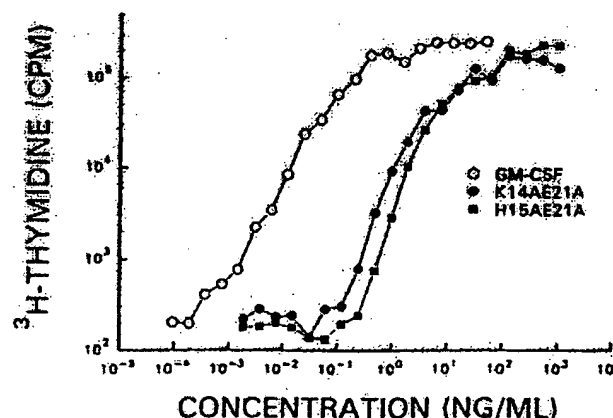


FIG. 2. Mutant bioactivity. The coding regions of mGM-CSF, K14AE21A, and H15AE21A were subcloned into the pET3A plasmid, expressed in the bacterial host strain BL21(DE3)pLysS (21), and purified to homogeneity using reverse-phase HPLC. 3 H-thymidine incorporation, reflecting DNA synthesis by the DA3 cell line (20), is presented graphically for each mutant and native mGM-CSF.

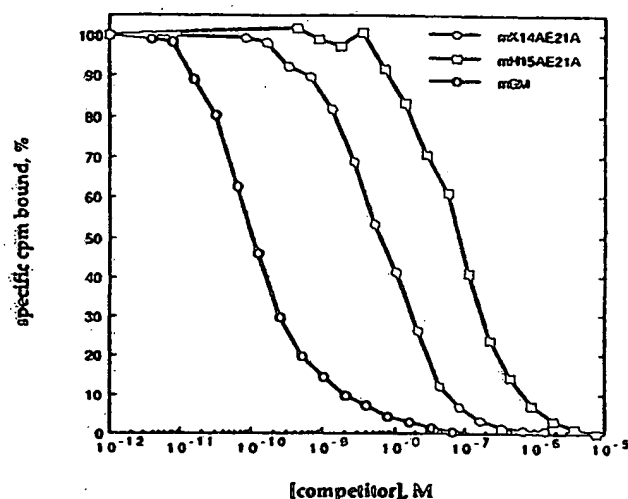


FIG. 3. Competitive binding of double mutants K14AE21A and H15AE21A. Competitive binding assays were performed with 2×10^6 acid-washed NFS60 cells using 30 pM ^{125}I -mGM-CSF. Both high and low affinity binding sites for mGM-CSF were detected. mGM-CSF (○), K_d -high = 58 pM (CV = 6%) and K_d -low = 7.5 nM (CV = 20%); K14AE21A (□), K_d = 5.0 nM (CV = 6%); H15AE21A (△), K_d = 62 nM (CV = 3%).

mutants has therefore been reduced at least 100- to 1000-fold, suggesting that this defect in binding is responsible for their loss of bioactivity.

DISCUSSION

The aim of these studies was to identify amino acid residues near the amino terminus of mGM-CSF that are necessary for cell surface receptor interaction and bioactivity. A multistep process was undertaken to screen alanine substitution mutants. Mutants were first selected based upon a loss of bioactivity and preservation of conformational integrity as defined by analysis of *N*-linked glycosylation. Finally, receptor-binding analysis was performed on the prokaryotic expression products of two mutants which had the greatest loss in bioactivity and a normal glycosylation pattern. Both K14AE21A and H15AE21A exhibited only a single binding site of low (5 and 62 nM , respectively) affinity. This represents a decrease of the (high affinity) dissociation constant of 100- to 1000-fold, indicating that the hydrophilic side chains of Lys-14, His-15, and Glu-21 are necessary for receptor binding and maximal bioactivity. It is interesting that H15AE21A has a dissociation constant 10-fold lower than K14AE21A, but has equivalent bioactivity; this paradox can be resolved only through further dissection of all of the components of the receptor/ligand complex. However, since the greatest reductions in specific activity are observed with double alanine

substitutions involving Glu-21, it is possible that this charged residue, in conjunction with Lys-14 and His-15, forms a dominant site of receptor-ligand interaction. The crystal structure of human GM-CSF has recently been reported (26). Consistent with prior predictions (7, 8, 16), an amino-terminal α -helix is present between residues 13 and 28. In this model, the side chains of Glu-14 and Glu-21 are exposed to solvent and comprise part of a putative receptor-binding site. The functional data presented here are consistent with these crystallographic findings, suggesting structural homology between murine and human GM-CSF in this region. Our work and the work of others have identified multiple regions of GM-CSF as being necessary for receptor binding, bioactivity, and species specificity (5, 6, 8-12, 14, 15); the present study defines the side chains in the amino-terminal α -helix that are critical for bioactivity.

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REFERENCES

1. Metcalf, D. (1985) *Science* 229, 16-22
2. Schrimsher, J. L., Rose, K., Simona, M. G., and Wingfield, P. (1987) *Biochem. J.* 247, 195-199
3. Clark, S. C., and Kamen, R. (1987) *Science* 236, 1229-1237
4. Gough, N. M., Metcalf, D., Gough, J., Grail, D., and Dunn, A. R. (1985) *EMBO J.* 4, 645-653
5. LaBranche, C. C., Clark, S. C., Johnson, G. D., Ornstein, D., Sebath, D. E., Tushinski, R., Paetkau, V., and Prystowsky, M. B. (1990) *Arch. Biochem. Biophys.* 278, 153-159
6. Shanafelt, A. B., and Kastelein, R. A. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 4872-4876
7. Gough, N. M., Grail, D., Gearing, D. P., and Metcalf, D. (1987) *Eur. J. Biochem.* 169, 353-358
8. Clark-Lewis, I., Lopez, A. F., To, L. B., Vadas, M. A., Schrader, J. W., Hood, L. E., and Kent, S. B. H. (1988) *J. Immunol.* 141, 881-889
9. Kaushansky, K., Shoemaker, S. G., Alfaro, S., and Brown, C. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 1213-1217
10. Brown, C. B., Hart, C. E., Curtis, D. M., Bailey, M. C., and Kaushansky, K. (1990) *J. Immunol.* 144, 2184-2189
11. Kanakura, Y., Cannistra, S. A., Brown, C. B., Nakamura, M., Seelig, G. F., Prosser, W. W., Hawkins, J. C., Kaushansky, K., and Griffin, J. D. (1991) *Blood* 77, 1033-1043
12. Altmann, S. W., Johnson, G. D., and Prystowsky, M. B. (1991) *J. Biol. Chem.* 266, 5333-5341
13. Meropol, N. J., Kreider, B. L., Lee, V. M.-Y., Kaushansky, K., and Prystowsky, M. B. (1991) *Hybridoma* 10, 433-447
14. Shanafelt, A. B., Johnson, K. E., and Kastelein, R. A. (1991) *J. Biol. Chem.* 266, 13804-13810
15. Shanafelt, A. B., Miyajima, A., Kitamura, T., and Kastelein, R. A. (1991) *EMBO J.* 10, 4105-4122
16. Parry, D. A. D., Minasian, E., and Leach, S. J. (1988) *J. Mol. Recogn.* 1, 107-110
17. Chothia, C. (1976) *J. Mol. Biol.* 105, 1-14
18. Rose, G. D., Geselovitz, A. R., Lesser, G. J., Lee, R. H., and Zehfus, M. H. (1985) *Science* 229, 834-838
19. Laemmli, U. K. (1970) *Nature* 227, 680-685
20. Ihle, J. N., and Keller, J. (1990) *Prog. Clin. Biol. Res.* 184, 85-94
21. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) *Methods Enzymol.* 185, 60-89
22. von Kimmensde, A., Bond, M. W., Schumacher, J. H., Lequai, C., and Kastelein, R. A. (1988) *Eur. J. Biochem.* 173, 109-114
23. Munson, P. J. (1983) *Methods Enzymol.* 92, 543-576
24. Ohtamoto, M., Naitai, M., Nakayama, C., Yanagi, H., Matsui, H., Noguchi, H., Namihira, M., Sakai, J., Kadota, K., Fukui, M., and Hara, H. (1991) *Arch. Biochem. Biophys.* 286, 562-568
25. Hayashida, K., Kitamura, T., Gorman, D. M., Arai, K., Yokota, T., and Miyajima, A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 9656-9659
26. Diederichs, K., Boone, T., and Karplus, A. (1991) *Science* 254, 1779-1782

High Affinity Ligand Binding Is Not Essential for Granulocyte-Macrophage Colony-stimulating Factor Receptor Activation*

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The high affinity receptor of the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) is a heterodimer composed of two members of the cytokine receptor superfamily. GM-CSF binds to the α -subunit (GM-R α) with low affinity and to the receptor $\alpha\beta$ complex (GM-R $\alpha\beta$) with high affinity. The GM-CSF-GM-R $\alpha\beta$ complex is responsible for biological activity. Interactions of the N-terminal helix of mouse GM-CSF with mGM-R $\alpha\beta$ were examined by introducing single alanine substitutions of hydrophilic residues in this region of mGM-CSF. The consequences of these substitutions were evaluated by receptor binding and biological assays. Although all mutant proteins exhibited near wild-type biological activity, most were defective in high affinity receptor binding. In particular, substitution of Glu-21 with alanine abrogated high affinity binding leaving low affinity binding unaffected. Despite near wild-type biological activity, no detectable binding interaction of this mutant with mGM-R β in the context of mGM-R $\alpha\beta$ was observed. Cross-linking studies showed an apparent interaction of this mutant protein with mGM-R $\alpha\beta$. The deficient receptor binding characteristics and near wild-type biological activity of this mutant protein demonstrate that mGM-CSF receptor activation can occur independently of high affinity binding, suggesting that conformational changes in the receptor induced by mGM-CSF binding generate an active ligand-receptor complex.

Granulocyte-macrophage colony-stimulating factor (GM-CSF)¹ is a cytokine that stimulates the growth and differentiation of several cell types of the hematopoietic system (1, 2). The cDNAs encoding the receptors for both human and mouse GM-CSF have been cloned and characterized (3-6); both receptors are heterodimers composed of subunits belonging to the cytokine receptor superfamily (7, 8). The α -subunit

(GM-R α) binds GM-CSF with low affinity ($K_d \sim 5 \times 10^{-9}$ M), whereas the complex of GM-R α and GM-R β forms the high affinity receptor (GM-R $\alpha\beta$; $K_d \sim 5 \times 10^{-11}$ M) (4, 5, 9). Although GM-R β does not bind GM-CSF in the absence of GM-R α , GM-CSF does interact directly with GM-R β in the context of the high affinity GM-R $\alpha\beta$ complex (10); formation of this complex containing GM-CSF is required for cellular signaling (4, 11).

The structures of human GM-CSF (12), human interleukin 4 (IL-4) (13, 14), and porcine growth hormone (GH) (15) have revealed a unique 4 α -helical bundle topology common to these cytokines. It is likely that mouse GM-CSF (mGM-CSF) is also a member of this structural family. Previous work has shown that residues in the N-terminal helix of GM-CSF are involved in high affinity binding to GM-R $\alpha\beta$ (10, 16, 17). We sought to determine the contribution of individual residues in this helix of mGM-CSF to mGM-R $\alpha\beta$ interaction. Mutant mGM-CSF proteins containing single alanine substitutions in the N-terminal helix exhibited near wild-type biological activity, but showed various defects in mGM-R $\alpha\beta$ binding. The mutation Glu-21 to alanine in mGM-CSF abrogated high affinity binding and binding interactions with mGM-R β in the ligand-mGM-R $\alpha\beta$ complex. The near wild-type activity of this mutant suggests that mGM-CSF receptor activation leading to cellular signaling can occur in the absence of high affinity ligand binding. Our findings suggest that activation of mGM-R $\alpha\beta$ may be induced by mGM-CSF binding to mGM-R α via mGM-R α to mGM-R β interactions, while high affinity binding complements this process to generate a fully active mGM-CSF-mGM-R $\alpha\beta$ complex.

MATERIALS AND METHODS

Bacterial Host Strains and Vectors—The *Escherichia coli* K12 strain JM101 (18) was used as host for the propagation and maintenance of M13 DNA. CJ236 (19) was used to prepare uracil-DNA for use in site-directed mutagenesis. AB1899 (20) was used as the host for expression of wild-type and mutant human and mouse GM-CSF proteins. pNIIIompH3 (21) was used as the expression vector for all GM-CSF genes. Elsewhere, we have described the expression of biologically active, mature GM-CSF with this *E. coli* secretory expression system (22).

Mutagenesis, Recombinant DNA, and Sequencing Protocols—Site-directed mutagenesis followed the protocol described by Kunkel *et al.* (19). Individual clones were sequenced using the dideoxynucleotide method (23) with modifications described in the Sequenase™ (U. S. Biochemical) protocol. M13 (replicative form) DNA containing correct mutations was cleaved with *Xba*I and *Bam*HI (New England Biolabs) for cloning into pNIIIompH3.

Preparation and Quantitation of Protein Extracts—Expression and quantitation of mutant proteins is described elsewhere (24). Briefly, all mutant proteins were produced in *E. coli* AB1899, and periplasmic extracts were prepared by osmotic shock (25). The specific activity of osmotic shock extracts varied <10% for a given mutant in multiple assays performed over several months' time. The amount of mutant polypeptide produced was determined using quantitative immunoblot.

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¹ The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; mGM-CSF, mouse GM-CSF; GM-R α , GM-CSF receptor α -subunit; GM-R β , GM-CSF receptor β -subunit; GM-R $\alpha\beta$, GM-CSF high affinity receptor; GH, growth hormone; hGH, human growth hormone; GH-R, growth hormone receptor; IL-2, -3, -4, and -6, interleukins 2, 3, 4, and 6; Epo, erythropoietin; HPLC, high-performance liquid chromatography; BS², bis(sulfosuccinimidyl)suberate.

blotting (24). The error in the calculated concentration of GM-CSF protein by this method was estimated to be 2-fold based on repetitive protein samples.

Mutant protein E21A was prepared to >98% purity (assessed by HPLC) from osmotic shock extracts (24) by affinity chromatography using agarose-coupled (AminoLink™, Pierce Chemical Co.) anti-mGM-CSF monoclonal antibody 35E10 (kindly provided by M. Pearce, DNAX Research Institute), followed by HPLC using a H₂O/acetonitrile gradient containing 0.1% trifluoroacetic acid using a Hi-Pore™ reversed-phase column (Bio-Rad). Purified E21A concentration was assessed by the absorption difference between 215 and 225 nm (26) compared to a purified mGM-CSF standard.

Biological Assays for Mouse GM-CSF Activity—Protein extracts were assayed using the mouse GM-CSF-dependent myeloid leukemia cell line NFS60. Sample concentrations were adjusted to 4000 pg/ml and titrated in quadruplicate to 0.02 pg/ml. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) assay described by Moesmann (27) was used to measure the extent of proliferation, and absorbance values were read with a V_{max} kinetic microplate reader (Molecular Devices). The concentration of each mutant and wild-type mGM-CSF protein that gave a 50% maximum response was determined using the Softmax program (Molecular Devices). The percent activity relative to wild-type mGM-CSF was calculated by the ratio of mGM-CSF EC₅₀/mutant EC₅₀ × 100%.

Purified mGM-CSF and E21A were tested in *in vitro* mouse whole bone marrow colony-forming unit, culture, assays as previously described (28). Seven-day colony counts in methyl cellulose were made for the combinations of mouse IL-3 (300 units/ml final; specific activity = 1×10^7 units/mg) + mouse IL-6 (250 units/ml final; specific activity = 5×10^6 units/mg) + mouse erythropoietin (Epo, 1 unit/ml final; purchased from Amgen); mGM-CSF (10 ng/ml final) + mouse Epo; and E21A (75 ng/ml final) + mouse Epo. Day 7 megakaryocyte colonies and eosinophil colonies in agar were done in the presence of mouse IL-3 + mouse IL-6; mouse IL-3 + mGM-CSF; and mouse IL-3 + E21A. The concentrations of mGM-CSF and E21A were chosen to give a maximal response based on the NFS60 proliferation assay. Assays were performed in triplicate.

Binding Assays of mGM-CSF Mutant Proteins—M43 was purified and labeled with ¹²⁵I as previously described (10). Mouse GM-CSF (obtained from Schering-Plough) was labeled with ¹²⁵I using the Bolton and Hunter reagent (Amersham; specific activity ~350–400 Ci/mmol). 3E6 (10) is an NFS60 cell line transfected with the human GM-Rα component (3). Binding assays were performed as follows: for competitive binding assays, NFS60 and 3E6 cells (maintained in mGM-CSF) were harvested and incubated with 1 ml of ice-cold 10 mM NaPO₄, 150 mM NaCl, pH 3.0, for 2 min, diluted to 50 ml with 10 mM NaPO₄, 150 mM NaCl, pH 7.0, centrifuged, and finally resuspended in 1 × Hanks' balanced salts solution (GIBCO-Bethesda Research Laboratories) containing 0.1% bovine serum albumin, 0.02% NaN₃, and 10 mM HEPES, pH 7.5 (HBAH buffer). 2×10^6 cells in 200-μl HBAH buffer were incubated with decreasing concentrations of unlabeled competitor in the presence of 30 pM ¹²⁵I-mGM-CSF or 10 pM ¹²⁵I-M43 (specific activity, 400 Ci/mmol) at 4 °C with continuous agitation for 4 h. Nonspecific binding was determined by including unlabeled mGM-CSF or M43 as appropriate at a concentration of 200 nM. Cell bound radioactivity was separated from free ligand by centrifugation at 4 °C (4 min, 12,000 × g) through diethylphthalate:dibutylphthalate (2:3), and bound and total radioactivity was measured with a Cobra 5010 γ-counter (Packard). The equilibrium binding data were analyzed using the Ligand program (29).

Scatchard analysis was performed as described above, except that NFS60 cells were used at 1×10^6 cells/point with decreasing concentrations of ¹²⁵I-mGM-CSF, or 1×10^5 and 5×10^6 cells/point with decreasing concentrations of ¹²⁵I-E21A (labeled with the Bolton and Hunter reagent (Amersham); specific activity, 1100 Ci/mmol) in quadruplicate. Nonspecific binding was assessed by including 1 μM mGM-CSF or E21A as appropriate.

Cross-linking of ¹²⁵I-mGM-CSF and ¹²⁵I-E21A to the mGM-CSF Receptor—2 nM labeled ligand (¹²⁵I-mGM-CSF, lanes 1, 3, 5, and 7; ¹²⁵I-E21A, lanes 2, 4, 6, and 8) was incubated with 2×10^6 NFS60 cells or 3E6 cells (maintained in human GM-CSF) for 4 h at 4 °C in HBAH buffer with (lanes 1, 2, 5, and 6) or without (lanes 3, 4, 7, and 8) 1 μM of the appropriate cold ligand. The cells were spun and treated with either BS³ (lanes 1–4; Pierce Chemical Co.) under conditions described in Ref. 9 or with 200 μM 1-ethyl-3-(3-diethylaminopropyl)carbodiimide, 1 mM sulfo-*N*-hydroxysuccinimide (lanes 5–8; Pierce Chemical Co.) in phosphate-buffered saline, pH 7.0, for 1 h at 23 °C. After exposure to cross-linking agent, cells were prepared

for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (30) as in Ref. 9, with equal counts per sample loaded and resolved on 8% gels. Dried gels were exposed to phosphorimaging screens for 3–4 days and developed using a Molecular Dynamics Phosphorimager.

RESULTS

Biological Response of mGM-CSF Mutant Proteins—Single alanine substitutions were introduced in the N-terminal helix of mGM-CSF by site-directed mutagenesis (19), replacing Lys-14 (mutant protein K14A), His-15 (H15A), Glu-17 (E17A), Lys-20 (K20A), and Glu-21 (E21A). mGM-CSF mutant protein was produced in *E. coli* and quantitated as previously described (24). Biological activity was considered a function of the EC₅₀ of each protein and was determined by proliferation of the mGM-CSF responsive mouse myeloid cell line NFS60 (Fig. 1). Proteins K14A, E17A, and K20A exhibited biological activity equal to or greater than wild-type mGM-CSF; proteins H15A and E21A exhibited at most a 3-fold reduction in biological activity, with no apparent change in the magnitude of the plateau. E21A mutant protein purified from *E. coli* extracts (>98% pure) was 29% as active as wild-type mGM-CSF (with respect to the EC₅₀) on NFS60 cells. Purified E21A also was tested in *in vitro* mouse bone marrow colony forming assays (28); saturating concentrations of E21A and wild-type mGM-CSF (based on the NFS60 assay) gave comparable values of both colony number and type (Table I).

Competitive Receptor Binding of mGM-CSF Mutant Proteins—The effects of amino acid substitutions on the ability of mGM-CSF to bind to mGM-Rα was assessed by competition binding assays using NFS60 cells. ¹²⁵I-mGM-CSF was used at a constant concentration of 3.0×10^{-11} M in the presence of various concentrations of unlabeled mGM-CSF or mutant protein extracts (Fig. 2). The receptor binding data obtained was analyzed using the Ligand program (29). Under these conditions, binding at both the low and high affinity sites for mGM-CSF on NFS60 cells was detected (Table II).

The amino acid residue substituted in a mutant protein that exhibited altered low affinity binding was considered to affect interactions, whether directly or indirectly, with mGM-Rα. Amino acid residues His-15 and Lys-20 were included in this class (Table II, mutant proteins H15A and K20A, respectively). High affinity binding of mGM-CSF to mGM-Rα is dependent on binding of mGM-CSF to mGM-Rα. Therefore, a decrease in affinity for mGM-Rα alone would cause a similar decrease in affinity for the high affinity mGM-Rα complex. The absolute value for the high affinity *K_d* thus may not

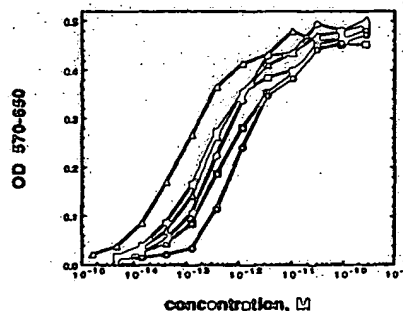


FIG. 1. Biological activity of wild-type and mutant mGM-CSF proteins. Proliferation of NFS60 cells in response to mGM-CSF (○) and mutant proteins K14A (Δ), H15A (□), E17A (◇), K20A (◻), and E21A (○) was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (27). Percent activities for the mutant proteins relative to the EC₅₀ of wild-type mGM-CSF were 140% for K14A, 64% for H15A, 550% for E17A, 210% for K20A, and 42% for E21A (mGM-CSF = 100%; EC₅₀ = 0.64 pM, standard deviation = 0.25 pM over replicate experiments).

TABLE I
Whole bone marrow colony-forming units (culture) assay of mGM-CSF and E21A

Cytokines ^a	Day 7 colonies in methyl cellulose ^a							
	GM	M	G	BFU-e	Mega	m-mixed	e-mixed	Total
IL-3 + IL-6 + Epo	198 ± 38	61 ± 18	87 ± 6	11 ± 3	13 ± 4	8 ± 4	19 ± 2	398 ± 46
mGM + Epo	136 ± 37	112 ± 32	46 ± 11	4 ± 1	0	0	1 ± 0.6	298 ± 76
E21A + Epo	144 ± 29	127 ± 7	45 ± 2	9 ± 2	0 ± 0.6	0	1 ± 0.6	325 ± 20

Cytokines ^a	Day 7 colonies in agar ^a	
	Mega colonies	Eosinophil colonies
IL-3 + IL-6	20 ± 3	13 ± 7
mGM	4 ± 1	10 ± 2
E21A	6 ± 2	11 ± 3

^a Assays were performed as described (28). Abbreviations: GM, granulocyte and macrophage colonies; M, macrophage colonies; G, granulocyte colonies; BFU-e, burst forming unit, erythroid; Mega, megakaryocyte colonies; m-mixed, mixed megakaryocyte colonies; e-mixed, mixed erythroid colonies. Values have error indicated at ± 1 S.D.

^b Cytokines used: mouse IL-3, 300 units/ml; mouse IL-6, 250 units/ml; Epo, 1 unit/ml mouse erythropoietin; mGM, 10 ng/ml mouse GM-CSF; E21A, 75 ng/ml.

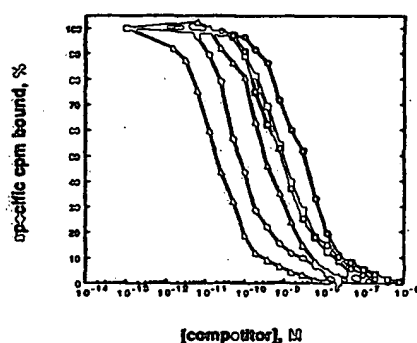


FIG. 2. Competitive binding of mutant and wild-type mGM-CSF proteins. Competition of 125 I-mGM-CSF on NFS60 cells by mGM-CSF (○), mutant K14A (Δ), H15A (□), E17A (◇), K20A (◻), and E21A (○). Assays were performed in triplicate with a constant concentration of 3.0×10^{-11} M 125 I-mGM-CSF. Under these conditions, both high and low affinity sites were detected using the Ligand program (29).

accurately represent the binding contribution due to mGM-CSF interaction with mGM-R β in the mGM-R $\alpha\beta$ complex. Effects on the interaction between mGM-CSF and mGM-R β in this context are therefore probably best described by the relative difference between low and high affinity binding constants. We chose to represent this difference as a change in the free energy, or $\Delta(\Delta G)$, between the two binding states (Table II). On this basis, interactions with mGM-R β were affected by alanine substitution of Lys-14, Lys-20, and Glu-21, but not by alanine substitution of His-15. Interestingly, changing Lys-20 to alanine appears to affect binding interactions with both GM-R α and GM-R β (Table II, K20A low affinity K_d and $\Delta(\Delta G)$, respectively). No binding defects were observed with the Glu-17 to alanine substitution (protein E17A).

Dissociation of mGM-R β Binding from mGM-R $\alpha\beta$.—In contrast to the other mutant proteins examined, E21A competed 125 I-mGM-CSF for only a single class of binding sites on NFS60 cells (Table II). The affinity of E21A for these sites suggests that binding is to mGM-R α ; competition under conditions that detected only mGM-R α binding (2 nM 125 I-mGM-CSF) also gave an equivalent affinity for E21A (data not shown). These observations were confirmed by receptor binding experiments using 125 I-E21A (displayed in Fig. 3 as a Scatchard plot). Two binding sites were statistically significant for wild-type mGM-CSF (Fig. 3A; $F = 78.9$ ($p = 0.0000$) for a one- versus a two-site fit); the mean K_d values were 26

TABLE II
Equilibrium binding constants and free energy contributions to high affinity binding of wild-type and mutant mGM-CSF proteins

Protein	K_d^a		$\Delta(\Delta G)^b$
	Low	High	
	nM	pM	kJ/mol
mGM-CSF	5.9	35	-11.8
K14A	3.8	180	-7.0
H15A ^c	77	450	-11.9
E17A	1.9	10	-12.1
K20A ^c	23	680	-8.1
E21A ^c	2.4	- ^d	0

^a Low and high refer to the low affinity and high affinity binding states as referred to in the text. Values were calculated using the Ligand program (29); the percent expression of the coefficient of variation (%CV, as described (29)) varied from 7 to 25% for the low affinity site and 9–12% for the high affinity site. The K_d values shown are from the statistically most reliable experiments. The low and high affinity K_d for mGM-CSF varied from 5.2 to 10.2 nM and 34 to 57 pM, respectively, in repetitive experiments.

^b The difference in free energy ($\Delta(\Delta G)$) quantitatively describes variations between the low and high affinity binding states. Since $\Delta G = -RT \ln K$, it follows that $(\Delta G_{\text{high affinity state}} - \Delta G_{\text{low affinity state}}) = \Delta(\Delta G) = -RT \ln(K_{\text{d-low}}/K_{\text{d-high}})$; comparison of this value for each mutant protein with that of wild-type mGM-CSF indicates the possible contribution of each residue to specifically mGM-R β interactions in the context of mGM-R $\alpha\beta$.

^c Values for the K_d for low affinity binding for these mutant proteins was confirmed by competition binding assays using 2 nM 125 I-mGM-CSF. Under these conditions only low affinity binding sites can be detected on NFS60 cells (data not shown).

^d Only a single, low affinity binding site was found to be statistically significant using the Ligand program ($F = 6.97$ ($p = 0.011$) for a one- versus a two-site fit) (29).

pM (coefficient of variation (CV) = 22%; 240 sites/cell) and 2.7 nM (CV = 46%; 4,000 sites/cell). In contrast, E21A exhibited only a single class of binding sites having a mean K_d value of 1.7 nM (Fig. 3B; CV = 26%, 3200 sites/cell). A two-site fit of the receptor binding data for 125 I-E21A was not statistically significant ($F = 2.24$ ($p = 0.14$) for a one- versus a two-site fit). Based on the affinity and the number of binding sites, E21A appears to bind to only mGM-R α . This suggested that E21A had lost its ability to interact with mGM-R β .

To rule out the possibility that a weak interaction between E21A and mGM-R β was being masked by low affinity binding to mGM-R α , a strategy was developed that allowed analysis of high affinity binding without interference from the low affinity binding site. The cell line used, 3E6 (10), is an NFS60 transfectant that in addition to having endogenous mGM-

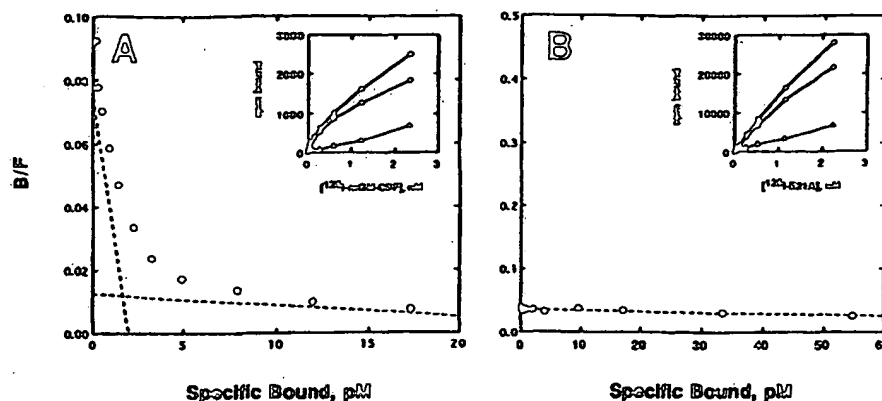


FIG. 3. Scatchard analysis of mGM-CSF and E21A binding to NFS60 cells. Representative experiments of ^{125}I -mGM-CSF (A) or ^{125}I -E21A (B) receptor binding to NFS60 cells. Five times more cells were used per data point for ^{125}I -E21A than for ^{125}I -mGM-CSF; the y-axis has been scaled such that the Scatchard transformations can be compared. Dashed lines represent the best fit values obtained from the Ligand program (29) for these experiments; K_d and binding site values were calculated by combining data from separate experiments. For mGM-CSF, both low and high affinity binding sites were detected, having mean K_d values of 2.7 nM (4000 sites/cell) and 26 pM (240 sites/cell), respectively (for a one- versus a two-site fit, $F = 78.9$ ($p = 0.0000$)). E21A exhibited only a single class of binding sites with a mean K_d of 1.7 nM (3200 sites/cell); a two-site fit was not statistically better than a one-site fit ($F = 2.24$, ($p = 0.14$)). Insets, the equilibrium binding isotherm (total bound (O), specific bound (O), and nonspecific bound (Δ)) ^{125}I -mGM-CSF or ^{125}I -E21A for the respective Scatchard plots are shown. The specific activity for ^{125}I -E21A was some 3-fold higher than ^{125}I -mGM-CSF; this, combined with the increased number of cells used for ^{125}I -E21A in this experiment, accounts for the higher absolute number of cpm bound for ^{125}I -E21A at comparable concentrations to ^{125}I -mGM-CSF.

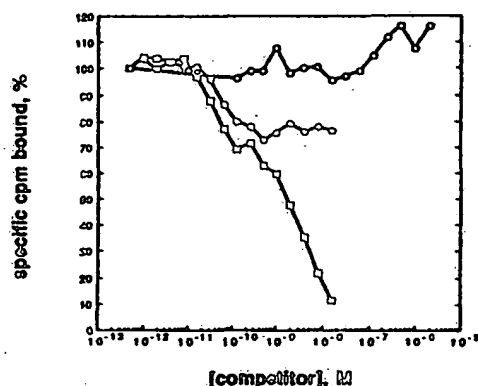


FIG. 4. Isolation mGM-CSF to mGM-R β interactions. Competition of ^{125}I -M43 on 3E6 cells by M43 (\square) (24), mGM-CSF (\circ), and E21A (\circ). Assays were performed in triplicate with a constant concentration of 1.0×10^{-11} M ^{125}I -M43, and the receptor binding data was analyzed with the Ligand program (29). M43 competes for both high and low affinity sites, while mGM-CSF competes for only the high affinity sites against ^{125}I -M43; E21A is unable to compete for any site. Competition of only the high affinity binding site of ^{125}I -M43 by mGM-CSF was observed in separate experiments. 3E6 (10) is an NFS60 cell line transfected with the human GM-R α component (3); M43 is a mouse/human GM-CSF hybrid protein consisting of the N-terminal 43 residues of mGM-CSF fused to the C-terminal 84 residues of hGM-CSF.

Ra β also expresses the low affinity component of the human GM-CSF receptor (hGM-R α) (3). M43 (24) is a mouse/human GM-CSF hybrid protein consisting of the N-terminal 43 residues of mGM-CSF fused to the C-terminal 85 residues of hGM-CSF. M43 binds to 3E6 with both low and high affinity, utilizing hGM-R α for low affinity binding, and hGM-R α together with mGM-R β for high affinity binding (10). Mouse GM-CSF competes specifically only for high affinity binding sites versus ^{125}I -M43 on 3E6 cells by forming high affinity complexes consisting of mGM-CSF-mGM-R β ; binding of ^{125}I -M43 to hGM-R α is unaffected (10).

Fig. 4 displays the results of a competitive binding assay on

3E6 cells using ^{125}I -M43. M43 is able to compete for both high and low affinity sites with mean K_d values of 22 pM (CV = 37%) and 3.0 nM (CV = 9%), respectively; mGM-CSF is able to compete only for the high affinity site with a mean K_d of 40 pM (CV = 40%) and shows no evidence of a second, low affinity site. E21A shows no evidence of competition for any site to a concentration of 2 μM , some 10^6 -fold higher than the wild-type K_d for high affinity binding. Since the affinity of this mutant protein for mGM-R α appears to be unaffected (Table II), this single amino acid substitution apparently abrogates specifically the binding interactions of E21A with mGM-R β in the context of mGM-Ra β .

Cross-linking of ^{125}I -E21A and ^{125}I -mGM-CSF—The association of E21A with mGM-Ra β was demonstrated by cross-linking ^{125}I -E21A (and ^{125}I -mGM-CSF) with the membrane impermeable form of disuccinimyl suberate, BS 3 (Fig. 5, lanes 1–4), or with 1-ethyl-3-(3-diethylaminopropyl)-carbodiimide (Fig. 5, lanes 5–8) to specific cell surface proteins on mGM-CSF-responsive cell lines. Using NFS60 cells in the presence of 2 nM ^{125}I -mGM-CSF, a specific band at the expected molecular weight of ~150 kDa for an ^{125}I -mGM-CSF-mGM-R β complex (6, 31) was observed with both BS 3 and 1-ethyl-3-(3-diethylaminopropyl)carbodiimide (Fig. 5A, lane 3, and faintly in lane 7, respectively). This band is not evident using ^{125}I -E21A (Fig. 5A, lanes 4 and 8). Identical results were obtained using 3E6 cells maintained in mGM-CSF (data not shown). However, the number of high affinity receptors (mGM-Ra β , and by implication mGM-R β) expressed on NFS60 cells is only about 240 per cell (Fig. 3). The low affinity of ^{125}I -E21A for mGM-Ra β may preclude detection of ^{125}I -E21A-mGM-R β complexes. The cell line 3E6 can be maintained in human GM-CSF; 3E6 cells so maintained express ~10-fold more high affinity mGM-CSF receptors than 3E6 cells maintained in mGM-CSF or NFS60 cells (data not shown). Using 3E6 cells maintained in human GM-CSF, the putative ^{125}I -mGM-CSF-mGM-R β complex appears as a more prominent band than with NFS60 cells (Fig. 5B, lanes 3 and 7). A band of comparable size is also seen with ^{125}I -E21A (lanes 4 and 8), suggesting the formation of an ^{125}I -E21A-

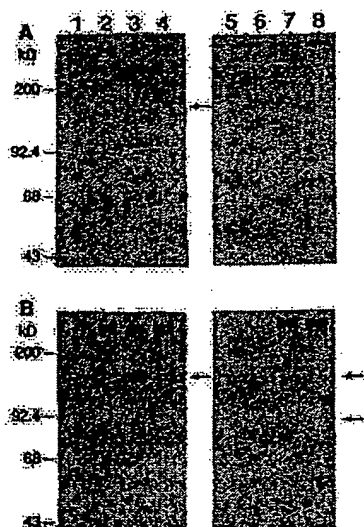


FIG. 5. Cross-linking of ^{125}I -mGM-CSF and ^{125}I -E21A to cell surface specific receptors. The products of *N*-hydroxysuccinimide-mediated (lanes 1-4) or carbodiimide-mediated (lanes 5-8) cross-linking of ^{125}I -mGM-CSF and ^{125}I -E21A were resolved by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. ^{125}I -mGM-CSF (lanes 1, 3, 5, and 7) or ^{125}I -E21A (lanes 2, 4, 6, and 8) was incubated with NFS60 cells or 3E6 cells (maintained in human GM-CSF) with (lanes 1, 2, 5, and 6) or without (lanes 3, 4, 7, and 8) 1 μM of the respective cold ligand. A, cross-linking to NFS60 cells; B, cross-linking to 3E6 cells maintained in human GM-CSF. Arrows at ~150 kDa indicate formation of the putative mGM-R β -ligand complex; molecular mass markers are indicated at left. A specific band possibly corresponding to a mGM-R α -ligand complex (~85 kDa, indicated by the lower arrow) was observed under carbodiimide mediated cross-linking conditions with ^{125}I -mGM-CSF and ^{125}I -E21A on 3E6 cells maintained in human GM-CSF (B, lanes 7 and 8).

mGM-R β complex. This suggests that E21A is proximal to mGM-R β when it binds to receptors on NFS60 cells. Since mGM-R β alone has no detectable affinity for mGM-CSF, cross-linking of ^{125}I -E21A to mGM-R β likely occurs through E21A interaction with mGM-R $\alpha\beta$.

DISCUSSION

Replacement of Glu-21 by alanine in mGM-CSF yielded a mutant protein, E21A, that had no high affinity binding nor detectable binding interaction with mGM-R β , yet was found to be in close proximity to mGM-R β by cross-linking studies in the context of mGM-R $\alpha\beta$. Strikingly, E21A had near wild-type mGM-CSF activity in stimulating the proliferation of responsive cell lines and inducing colony formation in *in vitro* whole bone marrow colony-forming assays. These results show that mGM-CSF receptor activation can occur in the absence of high affinity binding and independently of normal ligand binding interactions with mGM-R β in the context of mGM-R $\alpha\beta$. This suggests that receptor activation can be induced by mGM-CSF interaction only with mGM-R α in the mGM-R $\alpha\beta$ complex, and thus it is functionally independent of high affinity binding.

The crystal co-structure of human growth hormone (hGH), and its receptor (GH-R) has been solved (32). Interaction of hGH with its receptor occurs at two different regions of the hormone: site I interactions occur primarily through helix 4 of hGH with one GH-R subunit of the (GH-R) $_2$ homodimer, and site II interactions occur principally through helix 1 of hGH with the second GH-R subunit (32, 33). The interaction of GM-CSF with GM-R β in the context of GM-R $\alpha\beta$ (10, 16) appears to be similar to the site II interactions of GH and GH-R. The GH-R and GM-R $\alpha\beta$ subunits are members of the

same cytokine receptor family (8); therefore it seems likely that the structure of GM-R $\alpha\beta$ resembles (GH-R) $_2$. Assuming that this assumption is correct, a model of the GM-CSF-mGM-R $\alpha\beta$ complex can be proposed (Fig. 6). Helix 1 of GM-CSF is shown in contact with GM-R β . Following the topological pattern of the GH/(GH-R) $_2$ crystal structure, helix 4 of mGM-CSF is shown binding to GM-R α analogous to GH and GH-R site I interactions (32, 33) (Fig. 6A). The residues of mGM-CSF that we have found to affect mGM-R $\alpha\beta$ interactions are shown in their approximate position on the helix 1 backbone (Fig. 6B).

Competitive binding studies with each of these mutant proteins indicated that each substitution affected interactions with the subunits of mGM-R $\alpha\beta$. Changes in binding affinities due to mutational changes in a ligand can arise from direct or indirect effects (e.g. perturbations to the polypeptide backbone); in the absence of definitive structural information, absolute conclusions cannot be made. The high resolution crystal structure of human GM-CSF gives some insight into possible consequences of these mutations (12), and indicates the likely location of these residues on the helix 1 backbone (Fig. 6B). Substitution of Lys-14, Lys-20, and Glu-21 each affected interactions with mGM-R β . Each is located on the exterior of helix 1; given the structural similarities of GM-CSF and GH, these residues are situated for possible direct interactions with mGM-R β (Fig. 6B). Substitution of Lys-20 with alanine produced a mutant protein that also affected mGM-R α interactions. It is unclear from this study how this mutation can independently affect binding of the ligand with both receptor subunits. The substitution of His-16 affected mGM-R α binding and did not alter mGM-R β interactions (unchanged $\Delta(\text{AG})$ versus mGM-CSF, Table II). His-16 is buried in the core of the helical bundle in the crystal structure of hGM-CSF (12). Replacement of this residue by alanine may produce packing defects that indirectly deform the mGM-R α binding site, leaving the backbone structure of helix 1 intact. Further studies are needed to define the exact role that each of these residues plays in mGM-R $\alpha\beta$ interactions. Although Glu-17 is on the same surface of helix 1 as Lys-14 and

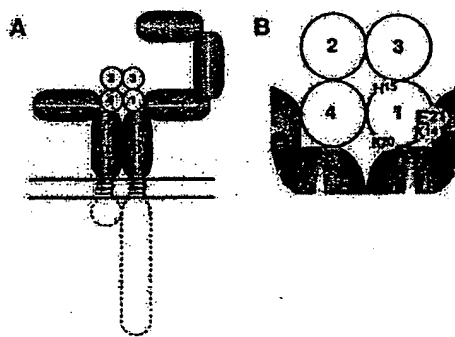


FIG. 6. Representation of GM-CSF to GM-R $\alpha\beta$ structural interactions. A, the GM-CSF ligand/receptor complex is shown based on the GH/GH-R cocrystal structure (32). The topology of the four helices of GM-CSF (circles, labeled 1 to 4) reflect a cross-section of the hGM-CSF crystal structure (12). Interactions of GM-CSF with GM-R β occur through helix 1 (10), similar to site II interactions in the GH-(GH-R) $_2$ complex (33). Analogous to site I GH to (GH-R) $_2$ interactions, it was assumed that helix 4 of GM-CSF is involved in mGM-R α binding. B, residues of mGM-CSF found in this study to be important to mGM-R $\alpha\beta$ interactions are placed in their relative locations along the helix 1 backbone. Replacement of Glu-21 by alanine resulted in the complete loss of ligand to GM-R β binding interactions in the context of GM-R $\alpha\beta$, and is highlighted to reflect this observation.

Glu-21, its replacement by alanine had no effect on mGM-R β interactions.

No high affinity binding was observed for E21A even though binding to mGM-R α was unaffected (Figs. 2 and 3B); direct interaction with mGM-R β in the context of mGM-R $\alpha\beta$ was apparently eliminated (Fig. 4). Surprisingly, only a modest decrease in biological activity was observed as a consequence of this substitution (Fig. 1 and Table I). E21A was shown to associate with the mGM-R $\alpha\beta$ complex by cross-linking ¹²⁵I-E21A on NFS60 cells (Fig. 5B), supporting the observation that signaling occurs only through a complex of the mGM-R α and mGM-R β subunits in the presence of ligand (4, 9).

The mitogenic response of NFS60 cells appears to be exquisitely sensitive to the presence of mGM-CSF. The K_d of mGM-CSF and number of high affinity receptors ($\sim 2.5 \times 10^{-11}$ M and ~ 240 mGM-R $\alpha\beta$ /NFS60 cell, respectively) suggest that only about 5–10 of these receptors are occupied at the EC_{50} ($\sim 6 \times 10^{-13}$ M). The K_d of E21A for mGM-R $\alpha\beta$ is only $\sim 2 \times 10^{-9}$ M, suggesting that ~ 20 -fold fewer mGM-R $\alpha\beta$ would be occupied at its EC_{50} ($\sim 2 \times 10^{-12}$ M). Receptor activation (indicated by mitogenic response) thus does not appear to be only a function of the affinity of the ligand for its receptor. The results obtained with E21A suggest that activation of mGM-R $\alpha\beta$ may occur through repeated binding and dissociation from the receptor complex or may induce an activated mGM-R $\alpha\beta$ complex that is capable of signaling without continuous ligand occupancy. Experiments that examine the kinetics of receptor interactions for E21A and the ability of mGM-R $\alpha\beta$ to internalize E21A would begin to address these possibilities.

The near wild-type potency and high affinity receptor binding defects of E21A suggest that mGM-CSF receptor activation can occur independently from mGM-CSF to mGM-R β interactions in the mGM-R $\alpha\beta$ complex. Consequently, binding of mGM-CSF to mGM-R α in the context of mGM-R $\alpha\beta$ appears to be the critical event in receptor activation. Since signaling is not observed in the absence of mGM-R β , a conformational change in mGM-R α induced by mGM-CSF binding producing a constructive interaction with mGM-R β would account for these results. Such an effect would not necessarily be limited to mGM-R α ; interaction of the mGM-CSF-mGM-R α complex with mGM-R β may also induce conformational changes in mGM-R β .

Although GM-CSF high affinity binding to both subunits of GM-R $\alpha\beta$ correlated well with a proliferative response (10), the results presented here show that receptor activation can occur independently of high affinity binding. However, small deviations in the sequence of mGM-CSF implicated in mGM-R $\alpha\beta$ binding interactions can profoundly affect biological activity. A mGM-CSF double mutant protein (both Lys-14 and Glu-21 changed to alanine) resulted in a 500-fold decrease in biological activity despite this protein having unchanged low affinity binding (17). It is possible that the presence of at least one of these residues is required for activation of mGM-R $\alpha\beta$. Alternatively, E21A bound to mGM-R $\alpha\beta$ may destabilize the receptor complex without disturbing its integrity, and additional mutations affecting receptor interactions may disrupt or prevent formation of the active complex. Other double mutants containing the Glu-21 to alanine substitution also had markedly reduced biological activity (including a His-15/Glu-21 to alanine mutant protein, but not a Glu-17/Glu-21 to alanine mutant protein) (17). Double mutant proteins without the Glu-21 to alanine substitution had less dramatic effects on bioactivity, suggesting a central role of Glu-21 in mGM-CSF to mGM-R β interactions.

The precise functional role of Glu-21 in high affinity mGM-

CSF to mGM-R $\alpha\beta$ interactions is not readily addressed by the experiments presented here. Qualitatively, it appears that the interaction of Glu-21 with mGM-R β allows other ligand-receptor contacts to form. In the E21A mutant protein, the potential for interactions exist in the form of the unchanged interactive residues (e.g. Lys-14 and Lys-20), but the substitution of Glu-21 with alanine appears to prevent binding (Fig. 4). About a 3-fold loss in biological activity (as compared to the EC_{50} of mGM-CSF) was observed for a purified preparation of E21A. This loss of activity suggests that high affinity binding contributes to receptor activation, but that binding energy alone is not the stimulus for receptor activation. It appears that an induced activation resulting from mGM-CSF binding to mGM-R α coupled to high affinity binding of mGM-CSF to mGM-R $\alpha\beta$ produces a fully active ligand-receptor complex.

An acidic residue (either Glu or Asp) equivalent to Glu-21 in GM-CSF is positionally conserved in the (predicted) N-terminal helices of a number of cytokines (10). Substitution of the acidic residue at this position in mouse (34) and human (35) IL-2 (Asp-34 and Asp-20, respectively) and human GM-CSF (16) (Glu-21) also had potent effects on receptor binding. In contrast to our results, these groups showed some correlation between high affinity receptor binding and bioactivity. This may be a reflection of the differences between the mGM-CSF and these other ligand-receptor systems, the cell types examined, the specific substitutions made, or combinations of these. The structural similarities between cytokines likely to have the GH and GM-CSF four α -helical bundle topology, and the potential similarities of their receptors make it attractive to consider that common mechanisms exist for ligand-receptor interactions. High affinity binding of cytokines and receptor subunit-subunit interactions induced by ligand binding may both be features of receptor activation.

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REFERENCES

- Gough, N. M., Metcalf, D., Gough, J., Grail, D., and Dunn, A. R. (1985) *EMBO J.* 4, 645–653.
- Metcalf, D. (1986) *Blood* 67, 257–267.
- Gearing, D. P., King, J. A., Gough, N. M., and Nicola, N. A. (1989) *EMBO J.* 8, 3667–3676.
- Hayashida, K., Kitamura, T., Gorman, D. M., Arai, K., Yokota, T., and Miyajima, A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 9655–9659.
- Parf, L. S., Martin, U., Sorensen, R., Lühr, S., Morrissey, P. J., Coaman, D., and Larsen, A. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 4295–4299.
- Gorman, D. M., Itoh, N., Kitamura, T., Schreum, J., Yonehara, S., Yohann, I., Arai, K., and Miyajima, A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 5459–5463.
- Icherdo, R. L., March, C. J., Mosley, B., Lyman, S. D., Vanden Boer, T., Gimpel, S. D., Din, W. S., Grubstein, K. H., Widmer, M. B., Parf, L. S., Coaman, D., and Beckmann, M. P. (1990) *J. Exp. Med.* 171, 861–873.
- Bazan, J. F. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 6934–6938.
- Kitamura, T., Sato, N., Arai, K., and Miyajima, A. (1991) *Cell* 66, 1165–1174.
- Shanafelt, A. B., Miyajima, A., Kitamura, T., and Kastelein, R. A. (1991) *EMBO J.* 10, 4105–4112.
- Kitamura, T., Hayashida, K., Sakamaki, K., Yokota, T., Arai, K., and Miyajima, A. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 5082–5086.
- Diederichs, K., Boone, T., and Karplus, P. A. (1991) *Science* 254, 1779–1782.
- Redfield, C., Smith, L. J., Boyd, J., Lawrence, G. M. P., Edwards, R. G., Smith, R. A. G., and Dobson, C. M. (1991) *Biochemistry* 30, 11029–11035.
- Smith, L. J., Redfield, C., Boyd, J., Lawrence, G. M. P., Edwards, R. G., Smith, R. A. G., and Dobson, C. M. (1992) *J. Mol. Biol.* 224, 899–904.
- Abdel-Meguid, S. S., Shieh, H., Smith, W. W., Dayringer, H. E., Vieland, B. N., and Bentle, L. A. (1987) *Proc. Natl. Acad. Sci. U. S. A.* 84, 6434–6437.
- Lopez, A. F., Shannon, M. F., Hercus, T., Nicola, N. A., Cambareri, B., Dottors, M., Layton, M. J., Eginton, L., and Vadas, M. A. (1992) *EMBO J.* 11, 909–916.
- Meropol, N., Altmann, S. W., Shanafelt, A. B., Kastelein, R. A., Johnson, G. D., and Prystowsky, M. (1992) *J. Biol. Chem.* 267, 14266–14269.
- Messing, J. (1983) *Methods Enzymol.* 101, 20–78.
- Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol.* 154, 367–382.

20. Howard-Flanders, P., Simson, R., and Theriot, L. (1964) *Genetics* 49, 237-246
21. Lundell, D., Greenberg, R., Alroy, Y., Condon, R., Fossetta, J. D., Gewain, K., Kastelein, R., Lunn, C. A., Reim, R., Shah, C., Van Kimmenade, A., and Narula, S. K. (1990) *J. Indust. Microbiol.* 5, 215-228
22. Greenberg, R., Lundell, D., Alroy, Y., Bonitz, S., Condon, R., Fossetta, J., Frommer, B., Gewain, K., Katz, M., Leibowitz, P. J., Narula, S. K., Kastelein, R., and Van Kimmenade, A. (1988) *Curr. Microb.* 17, 321-332
23. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* 74, 5463-5467
24. Shanafelt, A. B., Johnson, K. E., and Kastelein, R. A. (1991) *J. Biol. Chem.* 266, 13804-13810
25. Shanafelt, A. B., and Kastelein, R. A. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 4872-4876
26. Segel, I. H. (1976) *Biochemical Calculations*, Vol. 334, John Wiley and Sons, New York
27. Mosmann, T. (1983) *J. Immunol. Methods* 65, 55-63
28. Rennick, D., Yang, G., Muller-Sieburg, C., Smith, C., Arai, N., Takabe, Y., and Gemmell, L. (1987) *Proc. Natl. Acad. Sci. U. S. A.* 84, 6889-6893
29. Munson, P. J. (1983) *Methods Enzymol.* 92, 543-576
30. Laemmli, U. K. (1970) *Nature* 227, 680-685
31. Itoh, N., Yonehara, S., Schreurs, J., Gorman, D. M., Maruyama, K., Ishii, A., Yahara, I., Arai, K., and Miyajima, A. (1990) *Science* 247, 324-327
32. de Voe, A. M., Ultsch, M., and Kossiakoff, A. A. (1991) *Science* 255, 306-312
33. Cunningham, B. C., Ultsch, M., De, V. A., Mulkerrin, M. G., Clauser, K. R., and Wells, J. A. (1991) *Science* 254, 821-825
34. Zurawski, S. M., and Zurawski, G. (1988) *EMBO J.* 7, 1061-1069
35. Arima, N., Kamio, M., Okuma, M., Ju, G., and Uchiyama, T. (1991) *J. Immunol.* 147, 3396-3401

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A Role for the Carboxyl Terminus of Human Granulocyte-Macrophage Colony-stimulating Factor in the Binding of Ligand to the α -Subunit of the High Affinity Receptor*

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A synthetic segment (110–127) of the carboxyl terminus of recombinant human granulocyte-macrophage colony-stimulating factor (rh-GM-CSF) was used to generate a rabbit polyclonal antibody (345-6), which recognized both peptide and full-length *Escherichia coli*-derived rh-GM-CSF in a direct enzyme-linked immunosorbent assay. Antibody 345-6 was shown to antagonize the binding of 125 I-labeled rh-GM-CSF to its receptor on the KG-1 cell line and to inhibit human GM-CSF-dependent proliferation of the AML-193 cell line. The purified IgG fraction of neutralizing antibody 345-6 was used as immunogen to obtain sheep antiserum 1418. Antibody 1418 recognized antibody 345-6 on direct enzyme-linked immunosorbent assay but did not recognize rh-GM-CSF or the peptide 110–127 to which antibody 345-6 was raised. Antiserum 1418, as well as a purified IgG fraction of this serum, inhibited both rh-GM-CSF-stimulated cell proliferation and 125 I-labeled rh-GM-CSF receptor binding but not 125 I-labeled recombinant human interleukin-4 receptor binding. The anti-idiotypic antibody response derived from the anti-(110–127) antibody strongly suggests that the carboxyl-terminal region of rh-GM-CSF may be directly involved in the receptor-ligand interaction of this protein. The high affinity receptor consists of two different components (GM-Ra β) a cytokine-specific α -subunit and a β -subunit that is shared by human GM-CSF, interleukin-3, and interleukin-5. In an effort to localize the epitope of antibody 1418 to either GM-Ra or GM-R β , several cell lines containing high, low, or both high and low affinity receptors were examined. Each was specifically and completely inhibited by antibody 1418. Interleukin-3-dependent cell proliferation of the AML-193 cell line was found to be unaffected by the antibody 1418. Thus, the carboxyl-terminal region of rh-GM-CSF is likely to be involved in the interaction of the ligand with the α -subunit of the high affinity receptor.

Human granulocyte-macrophage colony-stimulating factor (h-GM-CSF)¹ is a member of a family of glycoproteins that stimulates growth and differentiation of a number of cell

types of hematopoietic lineage (Metcalf, 1986; Clark and Kamen, 1987). Clinical trials with rh-GM-CSF derived from mammalian and bacterial hosts (Greenberg *et al.*, 1988; Trotta, 1989) have been initiated in a variety of disease states, including acquired immunodeficiency syndrome (Groopman *et al.*, 1987; Baldwin *et al.*, 1988), myelodysplastic syndrome (Vadhan-Raj *et al.*, 1987), bone marrow transplantation (Monroy *et al.*, 1987), and in combination with chemotherapy in cancer patients (Brandt *et al.*, 1988).

The actions of h-GM-CSF are mediated through binding to cell-surface receptors (Gasson *et al.*, 1986; DiPersio *et al.*, 1988). The high affinity receptor is believed to be a heterodimer consisting of both a cytokine-specific α -subunit and a β -subunit that is shared with IL-3 and IL-5 (Hayashida *et al.*, 1990). The α -subunit of the receptor binds to the h-GM-CSF with low affinity (Gearing *et al.* 1989). Formation of the high affinity receptor occurs only in the complex of h-GM-CSF with both the α - and β -subunits. While the β -subunit does not itself bind to h-GM-CSF in the absence of the α -subunit, it does appear to interact directly with h-GM-CSF in the complex (Shanafelt *et al.*, 1991a).

Significant insight to the specific residues on mouse GM-CSF in contact with the β -subunit of the receptor in the high affinity receptor-ligand complex has been provided by Shanafelt *et al.* (1991b) and by Shanafelt and Kastelein (1992). In addition, critical cytoplasmic domains of the β receptor for growth signal transduction and tyrosine phosphorylation have been elucidated by Sakamaki *et al.* (1992). The residues on h-GM-CSF that may be involved in the binding of the α -subunit of the high affinity receptor are not yet known. In the present study, we sought to determine the domains of rh-GM-CSF that are involved in receptor interaction by raising both polyclonal antibodies to a synthetic peptide fragment and raising anti-idiotypic antibodies. The results support the conclusion that there is a segment at the carboxyl terminus of rh-GM-CSF that interacts directly with the cell-surface receptor. Further, the evidence provided suggests that it is likely to be the α -subunit of the receptor that binds to this terminal segment of h-GM-CSF.

EXPERIMENTAL PROCEDURES

Materials—Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD) and Jackson ImmunoResearch Laboratories (Avondale, PA) supplied horseradish peroxidase-conjugated goat anti-rabbit and donkey anti-sheep IgG, respectively. Calbiochem and DuPont NEN supplied monoclonal antibodies VIM-C6 and VIM-2 and 125 I-labeled Bolton-Hunter reagent (2000 Ci/mmol), respectively.

Cell Cultures and Reagents—The KG-1 cell line was obtained from the ATCC (CCL 246), and the AML-193 and TF-1 cell lines were provided to Schering-Plough Research Institute by Dr. G. Rovera (Lange *et al.*, 1987) and Dr. T. Kitamura of DNAX Research Institute, respectively. Placental membranes were prepared by homogenization in the presence of protease inhibitors, centrifugation to remove the

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¹ The abbreviations used are: h-GM-CSF, human granulocyte-macrophage colony-stimulating factor; rh-GM-CSF, recombinant human granulocyte-macrophage colony-stimulating factor; TBS, Tris-buffered saline; TBST, TBS containing 0.05% Tween; ELISA, enzyme-linked immunosorbent assay; AML, acute myeloid leukemia; IL, interleukin; IMDM, Iscove's modified Dulbecco's medium; FCS, fetal calf serum; Ab, antibody.

100 \times g fraction, and thorough washing of the 27,300 \times g fraction.

Cytokines—rh-GM-CSF (*Escherichia coli*, nonglycosylated; Schering-Plough/Sandoz) was purified to a constant maximal specific activity by methodology similar to that described for recombinant murine GM-CSF (Trotta *et al.*, 1987). Purified yeast-derived recombinant human IL-3 and purified Chinese hamster ovary cell-derived recombinant human IL-4 were prepared in the laboratories of the Schering-Plough Research Institute.

Preparation of Radiolabeled rh-GM-CSF—rh-GM-CSF was radioiodinated by the method of Bolton and Hunter (1973) and purified by gel filtration on a Sephadex G-25 column (PD-10, Pharmacia LKB Biotechnology Inc.). The resulting ^{125}I -labeled rh-GM-CSF had a specific radioactivity of $1\text{--}3 \times 10^6$ $\mu\text{Ci}/\mu\text{mol}$ and a stoichiometry of 0.4–1.2 mol of ^{125}I /mol of rh-GM-CSF. The specific radioactivity and stoichiometry were determined by the self-displacement method (Calvo *et al.*, 1983). ^{125}I -Labeled rh-GM-CSF exhibited the same level of biological activity in KG-1 and AML-193 cell proliferation assays as unlabeled GM-CSF.

Peptide Synthesis—The synthesis of peptides was carried out with an Applied Biosystems (Foster City, CA) model 430A fully automated peptide synthesizer (Merrifield, 1963). The amino-terminal *tert*-butyloxycarbonyl group was removed with 65% trifluoroacetic acid and cleaved from the phenylacetimido (polystyrene) resin with a 10:1.5 ratio of liquid hydrogen fluoride–anisole at 0 $^{\circ}\text{C}$ for 60 min. Cleaved, deprotected peptides were purified by reversed-phase high performance liquid chromatography on a C-4 Dynamax 400-Å wide bore column (Rainin Assoc., Woburn, MA). Automated sequencing and fast atom bombardment mass spectral analysis were employed to confirm the peptide sequence.

Production and Purification of Antisera—Rabbits were injected intradermally with an emulsion of 2 mg of antigen in 400 μl of TBS and 100 μl of pertussis vaccine and 500 μl of Freund's complete adjuvant. Boosts with incomplete adjuvant were scheduled when bleeds (as judged by ELISA) showed any loss of titer. The polyclonal antibody 1418 was produced in sheep by subcutaneous injection with 2.5 mg of an IgG fraction of rabbit antiserum 345-6. Sheep antibody 1418 was purified by Fortron Bioscience Co. (Saint Marys, PA) by ammonium sulfate precipitation and QAE ion exchange column chromatography.

ELISA—Rabbit and sheep sera were screened for specific binding of antigens by coating a 96-well microtiter plate (Becton-Dickinson) with 100 μl of antigen for 1 h at room temperature. Plates were washed with TBS containing 0.05% Tween 20 (TBST), blocked with 1% bovine serum albumin for 1 h, washed with TBST, blocked with 0.1% immunoglobulin for 1 h, and again washed with TBST. Blocking with immunoglobulin was omitted from the procedure for antibody 1418. The wells were coated with the antibody to be tested for 1 h, washed with TBST, then coated with 2.5 ng of horseradish peroxidase-conjugated goat anti-rabbit IgG or 5.0 ng of donkey anti-sheep IgG for 1 h, and washed with TBST. Development with either 2,2'-azino-bis(3-ethyl-benzthiazoline sulfonate) or 3,3',5,5'-tetramethyl benzidine and hydrogen peroxide was detected colorimetrically 20 min after the addition of enzyme substrates. Control wells were also developed in which one of the three assay components (i.e. antigen, antibody, peroxidase-labeled antibody) was deleted.

Receptor Binding Assay—Assays to measure ^{125}I -labeled rh-GM-CSF binding to receptors on KG-1 and AML-193 cells contained 50–100 pM ^{125}I -labeled rh-GM-CSF, $4\text{--}6 \times 10^5$ cells, and IMDM containing 10% FCS in a total volume of 0.4 ml. Samples were incubated at 4 $^{\circ}\text{C}$ for 2 h and centrifuged for 2.5 min at 600 \times g, and the cell pellet was washed twice with IMDM, 10% FCS and counted in a γ counter. Binding of ^{125}I -labeled rh-GM-CSF to receptors on placental membranes was quantitated by incubating membranes for 1 h at 22 $^{\circ}\text{C}$ with 0.5–5.0 nM ^{125}I -labeled rh-GM-CSF and IMDM, 10% FCS in a total volume of 0.4 ml, centrifuging for 2.5 min at 8000 g, washing the pellet with IMDM, 10% FCS, and counting. Saturating concentrations of unlabeled rh-GM-CSF were added to control assays to measure nonspecific binding. The data are presented as specific binding, which was determined by subtracting nonspecific from total ^{125}I -labeled rh-GM-CSF bound.

Competitive displacement of ^{125}I -labeled rh-GM-CSF from receptors by peptides or polyclonal antibodies was measured by including the competing ligand in binding assays. Antibodies recognizing rh-GM-CSF were incubated for 10 min at 4 $^{\circ}\text{C}$ with ^{125}I -labeled rh-GM-CSF prior to initiating binding by the addition of cells. Peptides and the sheep polyclonal antibody 1418 were incubated for 10 min at 4 $^{\circ}\text{C}$ with cells before ^{125}I -labeled rh-GM-CSF addition. Corresponding preimmune serum was also examined in control assays. None of the

rabbit or sheep preimmune sera (or purified commercially available rabbit or sheep IgG) interfered with GM-CSF receptor binding.

Cell Proliferation Assay—The assay for GM-CSF is based on stimulation of proliferation of KG-1 or AML-193 cells. The KG-1 is a cell line established from the bone marrow of a patient with AML (Lusis and Koefler, 1980; Koefler and Golde, 1978). The AML-193 is an acute childhood leukemia-derived cell line, which is dependent upon GM-CSF to support continued growth *in vitro* (Lange *et al.*, 1987). The TF-1 is a cell line established from a patient with erythroleukemia, which shows complete dependence on GM-CSF or IL-3 to support sustained growth (Kitamura *et al.*, 1989). Approximately 1×10^4 cells in IMDM containing 5 mg/ml each of insulin, transferrin, and sodium selenite were incubated in microtiter plate wells with dilutions of rh-GM-CSF for 6 days at 37 $^{\circ}\text{C}$ and then incubated for an additional 4 h with the tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (Mossmann, 1983). The results are expressed as a change in optical density (proportional to the log₂ cell concentration), which represents the difference in optical density of the sample and a base-line control lacking rh-GM-CSF.

Protein Concentration—Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard, unless otherwise noted.

RESULTS

Inhibition of Receptor Binding and Biological Activities by Anti-peptide Polyclonal Antibodies—In order to probe the regions of rh-GM-CSF that may be important to its receptor binding and biological activities, a series of peptides were synthesized (Table I). Rabbit polyclonal antibodies raised against each of these peptides were examined for their ability to bind peptide and rh-GM-CSF and to prevent binding of rh-GM-CSF to its receptor on KG-1 or AML-193 cells. Antiserum from one of these, anti-(110–127) antibody (Ab 345-6), shown by direct ELISA to bind both peptide 110–127 and rh-GM-CSF, was determined to completely inhibit receptor binding. In contrast, of the five other antisera that recognized (by ELISA) both the peptide to which they were raised and rh-GM-CSF, none blocked binding of rh-GM-CSF to receptor. Based on these data, we sought to further explore the role that this carboxyl-terminal segment might have in the actions of rh-GM-CSF.

In order to determine whether receptor and biological activities were specifically affected by this antibody, we purified the antiserum Ab 345-6 by protein A chromatography. The purified IgG blocked specific binding of ^{125}I -labeled rh-GM-CSF to its receptor on KG-1 cells in a concentration-dependent manner. Binding was completely prevented by preincubation of ^{125}I -labeled rh-GM-CSF with approximately 1.0 mg/ml of purified Ab 345-6 IgG. Half-maximal inhibition could be obtained using a concentration of 0.06 mg/ml (Fig. 1A). Nonimmunized rabbit serum and purified IgG had no effect on binding or nonspecific binding, respectively.

Next, the ability of purified Ab 345-6 to block rh-GM-CSF-stimulated proliferation of the AML-193 cell line was probed (Fig. 1B). Inhibition of proliferation was concentration-dependent, and 75% of rh-GM-CSF-dependent growth was prevented at 0.5 mg/ml IgG (the highest concentration examined). Half-maximal inhibition was obtained at 0.1 mg/ml in good agreement with the receptor binding inhibition observed. In the absence of rh-GM-CSF, no stimulation of AML-193 cell growth was observed. In addition, in the absence of rh-GM-CSF and the presence of sufficient quantities of Ab 345-6 to completely block rh-GM-CSF-stimulated proliferation of the AML-193 cell line, no change was observed in the AML-193 cell growth. Similarly, Ab 345-6 is shown to block rh-GM-CSF-stimulated proliferation of the TF-1 cell line under similar conditions (data not shown).

Inhibition of rh-GM-CSF by Anti-idiotypic Antibodies—We sought to determine whether the carboxyl terminus of rh-

TABLE I
Response of rabbit polyclonal antibodies raised to rh-GM-CSF and its peptides

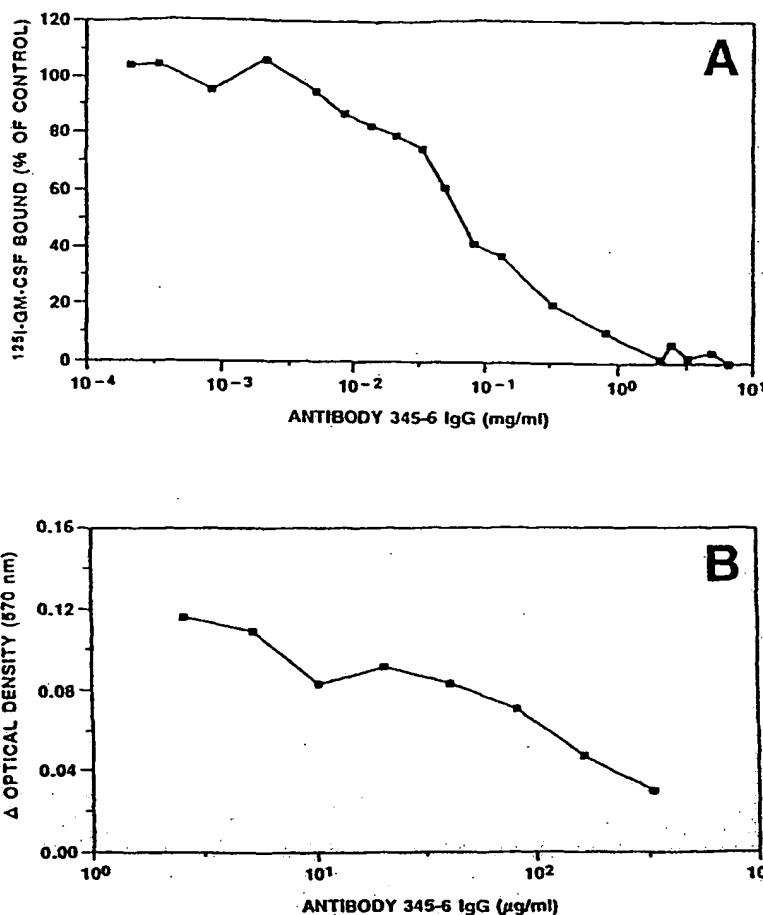
Antibody	Sequence no.	Sequence	Peptide response ^a	Protein response ^b	Inhibition
347-6	7-25 Cys	PSPSTQPWEHVNAIQEARRLC	90,000	90,000	—
346-6	Cys 26-41	CLNLSRDTAEMNETVE	8,000	8,000	—
351-6	45-54	EMFDLQEPTC	10,000	10,000	—
171-87	54-67	CLQTRLELYKQGLR	5,000	0	—
177-87	74-92	KGPLTMMASHYKQHCPPTP	100,000	0	—
137-88	94-119	TSCATQITTFESFKENLKDFLLVIPF	10,000	10,000	—
172-87	96-111	CATQITTFESFKENLK	1,000	1,000	—
345-6	110-127	LKDFFLLVIPFDCWEPVQE	10,000	10,000	+
349-6	GM-CSF	GM-CSF	NA	90,000	+

^a Recognition of peptide in a direct ELISA. Dilution gives 50% absorbance maximum. ELISA is coated with 0.25 μ g/well of peptide or protein. No response was observed for preimmune serum.

^b Recognition of rh-GM-CSF in a direct ELISA. Dilution gives 50% absorbance maximum as described above.

^c Details of the inhibition of ¹²⁵I-labeled rh-GM-CSF binding to KG-1 cells and GM-CSF-stimulated AML-193 cell proliferation by antibody Ab 345-6 are given in Fig. 1.

FIG. 1. Effect of purified rabbit IgG derived from antiserum 345-6 on receptor binding and *in vitro* biological activity of rh-GM-CSF. A, inhibition of ¹²⁵I-labeled rh-GM-CSF binding to KG-1 Cells. Assays contained 6×10^5 cells and 100 pM ¹²⁵I-labeled GM-CSF (21,000 cpm). Maximal specific binding in the absence of antibody was 2060 cpm. B, inhibition of GM-CSF-stimulated AML-193 cell proliferation. Assays contained 1×10^4 AML-193 cells and 10 ng/ml rh-GM-CSF. The increase in optical density (570 nm) in the absence of 345-6 IgG was 0.155.



GM-CSF could play a role in the ligand-receptor binding site of this cytokine. Peptide 110-127 itself was not an effective antagonist of rh-GM-CSF binding to KG-1 cells or agonist of rh-GM-CSF-independent cell proliferation of AML-193 cells (concentrations 10^6 -fold greater than the concentration of rh-GM-CSF were required to displace 50% of the radiolabeled ligand or stimulate proliferation). Therefore, we decided to explore alternative approaches to determine the role of the carboxyl terminus in the ligand-receptor binding site rh-GM-CSF. We chose to determine whether an anti-idiotypic antibody response could be generated using the polyclonal anti-110-127 antibody. Purified Ab 345-6 IgG was used to immunize a sheep, and the antiserum obtained (Ab 1418) recognized

the purified Ab 345-6 but not peptide 110-127 or rh-GM-CSF (Fig. 2).

Antiserum Ab 1418 was then tested for its ability to inhibit binding of ¹²⁵I-labeled rh-GM-CSF to AML-193 cells. The dose-dependent inhibition of binding by Ab 1418 is shown in Fig. 3A. Under the same conditions, preimmune serum had no effect. Ab 1418 had no effect on the nonspecific binding of ¹²⁵I-labeled rh-GM-CSF to the cells. The specificity of the interaction is further supported by evidence that demonstrates that Ab 1418 had no effect on the binding of ¹²⁵I-labeled IL-4 to receptors on Daudi cells (data not shown). Unpurified Ab 1418 was also tested for its ability to inhibit binding of ¹²⁵I-labeled rh-GM-CSF to KG-1 cells. A dose-

dependent inhibition curve for KG-1 cells was obtained, which was similar to that shown in Fig. 3A for AML-193 cells.

The ability of Ab 1418 to inhibit the rh-GM-CSF-dependent proliferation of AML-193 cells was examined using purified Ab 1418 IgG. Dose-dependent inhibition was obtained with complete inhibition being achieved with an IgG concentration of 1 mg/ml (Fig. 3B). Purified anti-idiotypic antibody Ab 1418 IgG was not able to stimulate proliferation of AML-193 even at suboptimal concentrations of rh-GM-CSF. It was also unable to stimulate AML-193 proliferation in the absence of added rh-GM-CSF. In similar experiments conducted with the KG-1 cell line, Ab 1418 serum was also shown to inhibit

rh-GM-CSF-dependent proliferation of the KG-1 cells in a dose-dependent manner (data not shown). Similar observations were made for the inhibition of rh-GM-CSF-stimulated proliferation of the TF-1 cell line by purified AB 1418.

Characterization of Neutralizing Anti-idiotypic Antibody 1418—The anti-idiotypic response obtained from the anti-110-127 antibody suggests that this antibody possesses the "internal image" and, thus, recognizes the rh-GM-CSF receptor. This receptor is believed to be a heterodimer consisting of an α -subunit, which is cytokine-specific, and a β -subunit, which is required for formation of the high affinity receptor. We wished to see if the epitope of the anti-idiotypic antibody Ab 1418 could be localized to the α - or β -subunit domains. We first attempted to see if Ab 1418 could inhibit the high and low affinity receptor activities. We hoped to distinguish the two by examining cell lines and receptor preparations that exhibit the low affinity α receptor and the high affinity α/β receptor (Table II). The KG-1 and AML-193 cell lines each display the high affinity receptor binding sites. While the KG-1 cell line also exhibits the low affinity receptor binding sites, no low affinity receptor binding site is observed for the AML-193 cell line. In contrast, both placental membranes and the choriocarcinoma-derived JAR cell line display only the low affinity receptor binding sites. These cell lines and membrane preparations, which appear to have high affinity sites, high and low affinity sites, or just low affinity sites are all completely and specifically inhibited by 1.4 mg/ml purified Ab 1418 IgG. This suggests that the α -subunit (GMR α) contains the Ab 1418 epitope, and, therefore, the

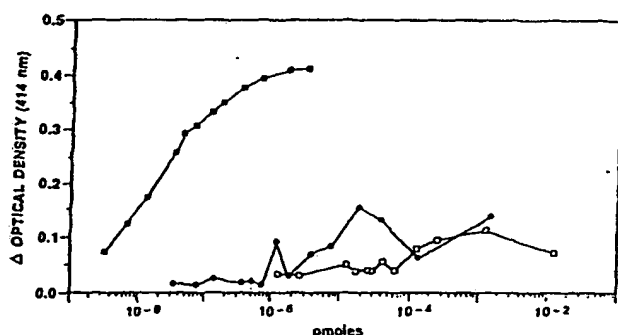


FIG. 2. Direct binding ELISA titration of purified rabbit IgG derived from antiserum 345-6 (■), rh-GM-CSF (●), and peptide 110-127 (□) by the purified IgG fraction of sheep antibody 1418.

FIG. 3. Effect of sheep antibody 1418 on receptor binding and *in vitro* biological activity of rh-GM-CSF. A, inhibition of 125 I-labeled rh-GM-CSF binding to AML-193 cells. Assays contained 6.4×10^5 cells, 20 pM 125 I-labeled rh-GM-CSF (17,400 cpm), and antiserum at the indicated dilutions. Specific binding in the absence of antiserum was 3180 cpm. B, inhibition of GM-CSF-dependent AML-193 cell proliferation. Assays contained 1×10^4 cells and 1.0 ng/ml rh-GM-CSF. The increase in absorbance at 570 nm in the absence of IgG was 0.270. (No stimulation of the AML-193 cells was observed in the absence of rh-GM-CSF.)

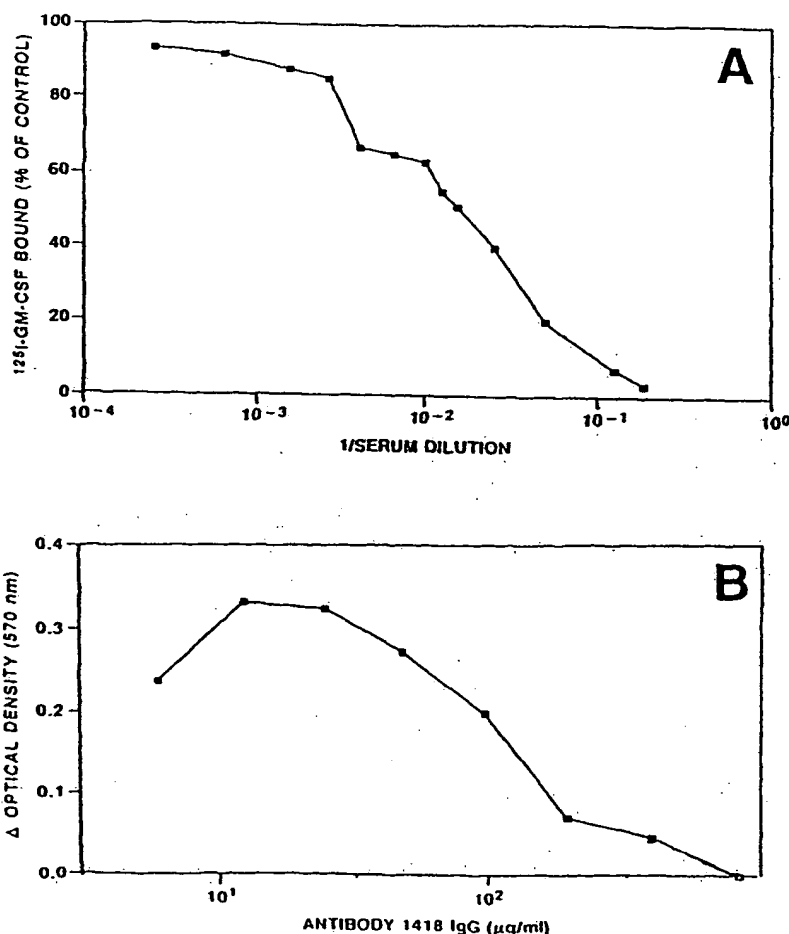


TABLE II
Inhibition of 125 I-labeled rh-GM-CSF binding to receptor by anti-idiotypic antibody Ab 1418

Cell type	K_d of high affinity site	K_d of low affinity site	Inhibition ^a
	μ M	nM	
AML-193	70		100
KG-1 ^b	6.7	0.73	100
JAR		2-4	100
Placental membranes		0.74	100

^a Maximal inhibition of 125 I-labeled rh-GM-CSF binding to cell-surface receptors by antibody 1418.

^b Two murine monoclonal antibodies (Madjic, 1984), one that was raised against KG-1 cells and that recognizes granulocytes (VIM-C6) and one that recognizes a carbohydrate determinant on human granulocytes, monocytes, and their precursors (VIM-2) were tested on KG-1 cells for inhibition of 125 I-labeled rh-GM-CSF binding to receptors. The fact that no inhibition was observed with either antibody further supports the specificity of the inhibition derived from the anti-idiotypic antibody 1418.

amino acid region containing residues 110-127 of h-GM-CSF is involved in the GMR α interaction.

We then wanted to confirm that Ab 1418 was targeting the α -subunit (GMR α). We chose to examine whether Ab 1418 would inhibit the IL-3-dependent proliferation of AML-193 cells. IL-3 shares the β -subunit (GMR β) with rh-GM-CSF. No inhibition of this biological activity was observed, suggesting that the β receptor subunit was not effected by this antibody and confirming that the involvement was occurring at the α -subunit.

DISCUSSION

In this study, we provide evidence for the importance of the carboxyl-terminal 18 amino acid residues of rh-GM-CSF in binding of the ligand to its cell-surface receptor and expression of biological activity. The anti-idiotypic antibody response underlines the importance of the carboxyl region and further suggests that this segment plays a direct role in the ligand-receptor binding site of rh-GM-CSF.

The high affinity receptor of h-GM-CSF heterodimer consists of the α -subunit, which confers cytokine specificity, and the β -subunit. The α -subunit alone displays low affinity for GM-CSF. The high affinity is displayed only when the β -subunit is also present. Both subunits are required for signal transduction. We demonstrate that the anti-idiotypic antibody Ab 1418 inhibits binding of rh-GM-CSF to both the high and low affinity receptor binding sites of several cell lines. This strongly indicates that the epitope for this antibody lies on the α -subunit. The fact that the Ab 1418 was derived from an antibody directed to a peptide corresponding to amino acids 110-127 of the rh-GM-CSF sequence suggests that the carboxyl terminus, or D helix, is responsible for the interactions of the cytokine with the α -subunit.

The high affinity receptors of h-GM-CSF, IL-3, and IL-5 are each heterodimers. In each case, the α -subunit is distinct and specific for the cytokine. The β -subunit, however, which is essential for signal transduction, is common to each. We confirmed that the β -subunit was not effected by Ab 1418 by examining the IL-3-dependent proliferation of the AML-193 in the presence of Ab 1418. In the case of IL-3, no inhibition could be detected.

The α -subunit of the h-GM-CSF receptor binds to h-GM-CSF with low affinity. While the β -subunit does not itself measurably bind to h-GM-CSF, it appears to interact directly with h-GM-CSF in the complex. Studies by Shanafelt *et al.* (1991a) provide evidence that the A, or amino-terminal, helix (residues 15-28) of the mouse GM-CSF governs the interac-

tion of the cytokine with the β or "shared" subunit. Using hybrid analysis and carboxyl-terminal deletions, Shanafelt *et al.* (1991b) demonstrated that significant losses of activity were incurred by human (but not mouse) GM-CSF. In particular, residue Trp-122 was acutely sensitive to substitution. Together, these data provide circumstantial evidence for an important role of the carboxyl terminus. In this paper, we provide direct evidence that a specific peptide region in the D, or carboxyl-terminal, helix (110-127) interacts specifically with the α -subunit. It is consistent that the x-ray crystallography of the four-helix bundle structure of h-GM-CSF (Walter *et al.*, 1992; Diederichs *et al.* 1991) suggests that helix B has very low solvent and helix C has moderate accessibility and that the amino helix A and the carboxyl helix D are solvent-exposed.

Thus, more than one region of rh-GM-CSF is implicated in expression of its varied activities. A role for more than one segment of a ligand in its interaction with receptor is consistent with a model of cytokine action exhibited by human growth hormone. The solution of the structure of the growth hormone co-crystal with its receptor provides information concerning the receptor-ligand binding sites within the complex (deVos *et al.*, 1992). Since this protein is believed to be in the same family as that of rh-GM-CSF, the structure may be a useful model of the complex that rh-GM-CSF forms with its receptor. Interaction of human growth hormone with its homodimer receptor occurs at two distinct regions of the ligand. The first site of interaction with receptor subunit 1 occurs at the first helix of the ligand. The second site of interaction occurs between the first and fourth helix of the ligand and receptor subunit 2. While the receptor subunits of rh-GM-CSF are not identical, a potentially similar pattern of interaction could be envisioned. Thus, the α receptor subunit of rh-GM-CSF would interact with the helix D or carboxyl terminus of the rh-GM-CSF ligand, whereas the β receptor subunit would interact with the first helix of the ligand as suggested by the studies with murine GM-CSF described by Shanafelt *et al.* (1992).

The development of the anti-idiotypic antibody Ab 1418, which interacts with the α receptor subunit, offers a unique opportunity to probe the heterodimer receptor of rh-GM-CSF. Antibody Ab 1418 provides an invaluable tool for defining the residues on the receptor that may be involved in the ligand-receptor binding site.

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REFERENCES

- Baldwin, G. C., Gasson, J. C., Quon, S. G., Fleischmann, J., Weisbart, R., Oette, D., Mitsuyasu, R. T., and Golde, D. W. (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85, 2763-2766.
- Bolton, A. E., and Hunter, W. M. (1973) *Biochem. J.* 133, 529-539.
- Brandt, S. J., Peters, W. P., Atwater, S. K., Kurtzberg, J., Borowitz, M. J., Jones, R. B., Shpall, E. J., Bant, R. C., Gilbert, C. J., and Oette, D. J. (1988) *New Engl. J. Med.* 318, 869-876.
- Calvo, J. C., Radicella, J. P., and Charreau, E. H. (1983) *Biochem. J.* 212, 259-264.
- Clark, S. C., and Kamen, R. (1987) *Science* 236, 1229-1237.
- deVos, A. M., Ullsch, M., and Kossiakoff, A. A. (1992) *Science* 255, 308-312.
- Diederichs, K., Jacquet, S., Boone, T., and Karplus, P. A. (1991) *J. Mol. Biol.* 221, 55-60.
- DiPersio, J., Billing, P., Kaufman, S., Eghtesady, P., Williams, R. E., Gasson, J. C. (1988) *J. Biol. Chem.* 263, 1834-1841.
- Gasson, J. C., Kaufman, S. E., Weisbart, R. H., Tomonaga, M., and Golde, D. W. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 669-673.
- Gearing, D. P., King, J. A., Gough, N. M., and Nicola, N. A. (1989) *EMBO J.* 8, 3667-3676.
- Greenberg, R., Lundell, D., Alroy, Y., Bontz, S., Condon, R., Fossetta, J., Frommer, B., Gewain, K., Katz, M., Leibowitz, P. J., Narula, S. K., Kastelein, R., and Van Kimmene, A. (1988) *Curr. Microbiol.* 17, 321-332.
- Groopman, J. E., Mitsuyasu, R. T., DeLeo, M. J., Oette, D. H., and Golde, D. W. (1987) *New Engl. J. Med.* 317, 593-598.
- Hayashida, K., Kitamura, T., Gorman, D. M., Arai, K., Yokota, T., and Miyajima, A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 9655-9659.

- Kitamura, T., Tange, T., Terasawa, T., Chiba, S., Kuwaki, T., Miyagawa, K., Piao, Y.-F., Miyazono, K., Urabe, A., and Takaku, F. (1989) *J. Cell. Physiol.* **140**, 323-334.
- Koeffler, H. P., and Golde, D. W. (1978) *Science* **200**, 1153-1154.
- Lange, B., Valtieri, M., Santoli, D., Caracciolo, D., Mavilio, F., Gemperlein, L., Griffin, C., Emanuel, B., Finan, J., Nowell, P., and Rovera, G. (1987) *Blood* **70**, 192-199.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275.
- Lusia, A. J., and Koeffler, H. P. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 5346-5350.
- Madje, O., Bettelheim, P., Stockinger, H., Aberer, W., Liska, K., Lutz, D., and Knapp, W. (1984) *Int. J. Cancer* **33**, 617-623.
- Merrifield, R. B. (1963) *J. Am. Chem. Soc.* **85**, 2149-2154.
- Metcalfe, D. (1986) *Blood* **67**, 257-267.
- Monroy, R. L., Skelly, R. R., MacVittie, T. J., Davis, T. A., Scuber, J. J., Clark, S. C., and Donahue, R. E. (1987) *Blood* **70**, 1096-1099.
- Mosmann, T. (1983) *J. Immunol. Methods* **65**, 55-63.
- Sakamaki, K., Miyajima, I., Kitamura, T., and Miyajima, A. (1992) *EMBO J.* **11**, 3541-3549.
- Shanafelt, A. B., and Kastelein, R. A. (1992) *J. Biol. Chem.* **267**, 25466-25472.
- Shanafelt, A. B., Miyajima, A., Kitamura, T., and Kastelein, R. A. (1991a) *EMBO J.* **10**, 4105-4112.
- Shanafelt, A. B., Johnson, K. E., and Kastelein, R. A. (1991b) *J. Biol. Chem.* **266**, 13804-13810.
- Trotta, P. P. (1989) in *Proceedings of the Satellite Symposium at the 9th European Conference on Clinical Oncology* (Salmon, S., ed) pp. 6-8, Peninine Press, Bollington, United Kingdom.
- Trotta, P. P., Sealig, G. F., Kosecki, R. A., and Reichert, P. (July 16, 1987) European Patent Application 88306421.4.
- Vandan-Raj, S., Keating, M., LeMaistre, A., Hittleman, W. N., McCredie, K., Trujillo, J. M., Broxmeyer, H. E., Henny, C., and Gutterman, J. (1987) *New Engl. J. Med.* **317**, 1545-1551.
- Walter, M. R., Cook, W. J., Elick, S. E., Nagabhushan, T. L., Trotta, P. P., and Bugg, C. E. (1992) *J. Mol. Biol.* **224**, 1075-1085.

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The amino-terminal helix of GM-CSF and IL-5 governs high affinity binding to their receptors

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Transduction of the biological effects of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-5 (IL-5) requires the interaction of each cytokine with at least two cell surface receptor components, one of which is shared between these two cytokines. A strategy is presented that allowed us to identify receptor binding determinants in GM-CSF and IL-5. Mixed species (human and mouse) receptors were used to locate unique receptor binding domains on a series of human–mouse hybrid GM-CSF and IL-5 cytokines. Results show that the interaction of these two cytokines with the shared subunit of their high affinity receptor complexes is governed by a very small part of their peptide chains. The presence of a few key residues in the amino-terminal α -helix of each ligand is sufficient to confer specificity to the interaction. Comparison with other cytokines suggests that the amino-terminal helix of many of these proteins may contain the recognition element for the formation of high affinity binding sites with the α subunit of their multi-component receptors. **Key words:** cytokines/mutagenesis/receptors/structure–function

Introduction

The biological effects of cytokines on cell growth and differentiation are mediated by specific cell surface molecules. These molecules transduce the binding of their cognate cytokines into cytoplasmic signals that eventually trigger a cascade of intracellular responses. GM-CSF is a cytokine that stimulates the development of various lineages of hemopoietic cells (Gough and Nicola, 1989; Metcalf, 1986). Hayashida *et al.* (1990) have shown that its functional receptor is composed of at least two subunits, GM-CSF receptor α (GM-R α) and GM-CSF receptor β (GM-R β) (Chiba *et al.*, 1990; Hayashida *et al.*, 1990; Kitamura *et al.*, 1991a). The α subunit binds GM-CSF with low affinity, whereas the β subunit does not measurably bind GM-CSF. Coexpression of GM-R α and -R β leads to high affinity binding of GM-CSF and is required for signal transduction (Hayashida *et al.*, 1990). Recent evidence indicates that the GM-R β subunit is shared with the receptor systems of other cytokines (Hayashida *et al.*, 1990; Kitamura *et al.*, 1991a). High affinity binding of human interleukin-3 (IL-3), IL-5 and GM-CSF appears to require the common GM-R β subunit in combination with a receptor subunit that is unique

for each of these cytokines (Hayashida *et al.*, 1990; Kitamura *et al.*, 1991b; Lopez *et al.*, 1990). In mouse, GM-CSF and IL-5 receptors share a common β subunit (AIC2B) (Devos *et al.*, 1991; Gorman *et al.*, 1990; Itoh *et al.*, 1990; Kitamura *et al.*, 1991a), while IL-3 incorporates the homologous AIC2A molecule in its high affinity receptor complex (Schreurs *et al.*, 1991). These findings provide an explanation for earlier observations indicating that these cytokines can compete with one another, either completely or partially (Budel *et al.*, 1990; Lopez *et al.*, 1990; Park *et al.*, 1989a,b).

Binding of GM-CSF to its functional receptor is followed by transduction of a signal to unknown cytoplasmic messengers. To understand the mechanism of signal transduction in a receptor system of such complexity, it is essential to identify the molecular nature of the cytokine–receptor interaction. Human and mouse GM-CSF are species specific; we have used this property to search for unique receptor binding domains in this protein hormone. To approach this problem, human–mouse GM-CSF hybrid proteins were analyzed in combination with mixed species human and mouse GM-CSF receptors. High affinity association (and a concomitant biological response) of mixed species α and β GM-CSF receptor chains is only expected to occur when hybrid GM-CSF cytokines present the correct species-specific receptor binding domains. This approach allowed us to identify the residues of the cytokine that interact with the β subunit of the receptor. These amino acids lie in the amino-terminal α -helix of GM-CSF.

Using a mouse(m)IL-5–human(h)GM-CSF hybrid we also show that mIL-5 interacts with GM-R β through residues in its amino-terminal α -helix. Comparative analysis of other receptor–cytokine pairs leads us to propose that the amino-terminal α -helix of many cytokines is the segment that is recognized by a component of their functional receptors.

Results

Mixed species receptors

The mGM-CSF dependent myeloid cell line NFS60 was stably transfected with a plasmid encoding the α subunit of the human GM-CSF receptor, and was named 3E6. Whereas NFS60 does not proliferate in response to hGM-CSF, 3E6 responds weakly to hGM-CSF. This presumably occurs through an interaction of the hGM-CSF–hGM-R α complex with the mGM-R β component (Kitamura *et al.*, 1991a). However, no high affinity binding sites for hGM-CSF were observed on this cell line (data not shown). We had previously constructed a collection of human–mouse GM-CSF hybrid proteins (Shanafelt *et al.*, 1991). A comparison of the human and mouse GM-CSF amino acid sequences and a pictorial representation of the hybrid proteins are displayed in Figure 1. The responses of NFS60 and 3E6 to the series of amino-terminal mouse–carboxy-terminal human GM-CSF hybrids is shown in Figure 2a. The

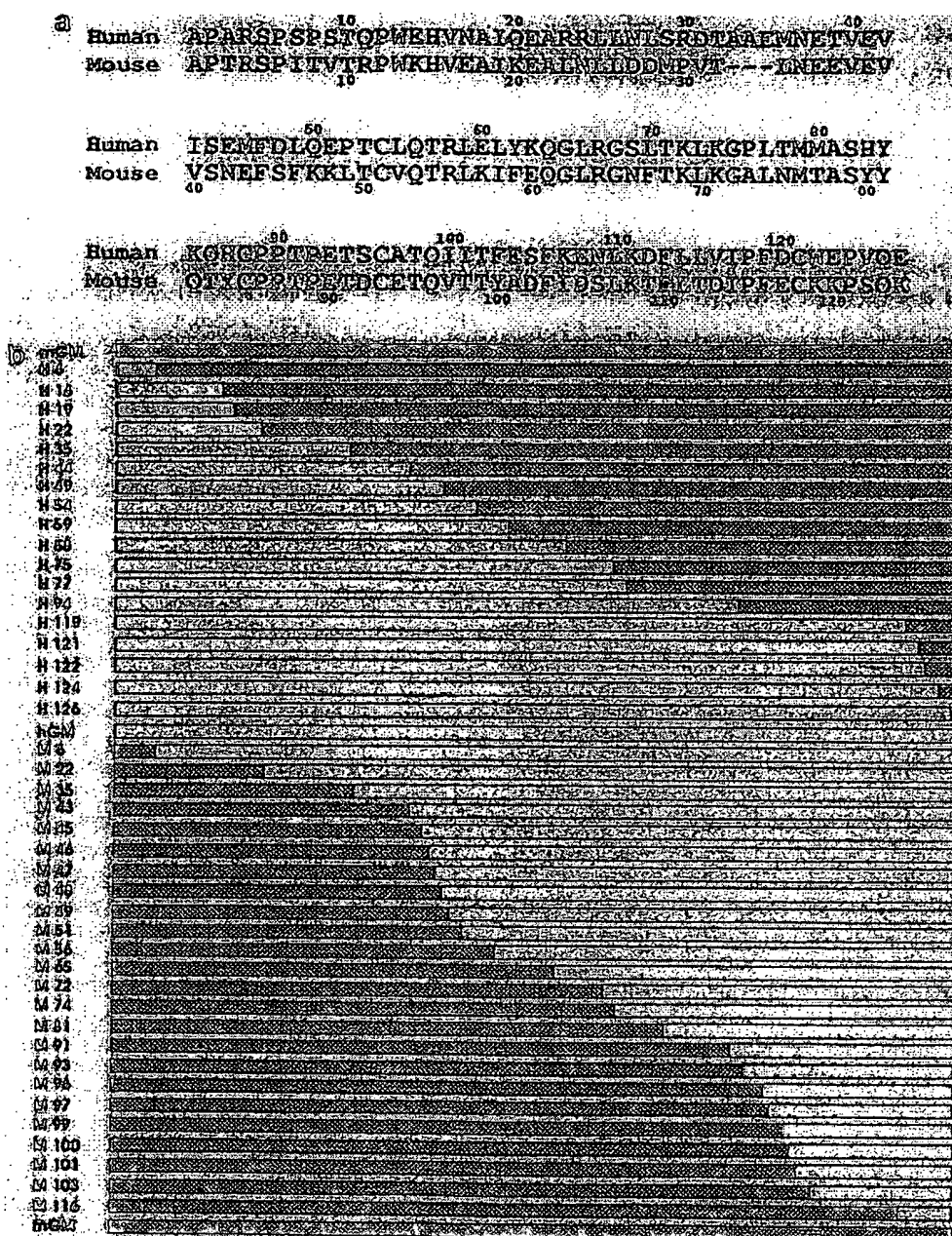
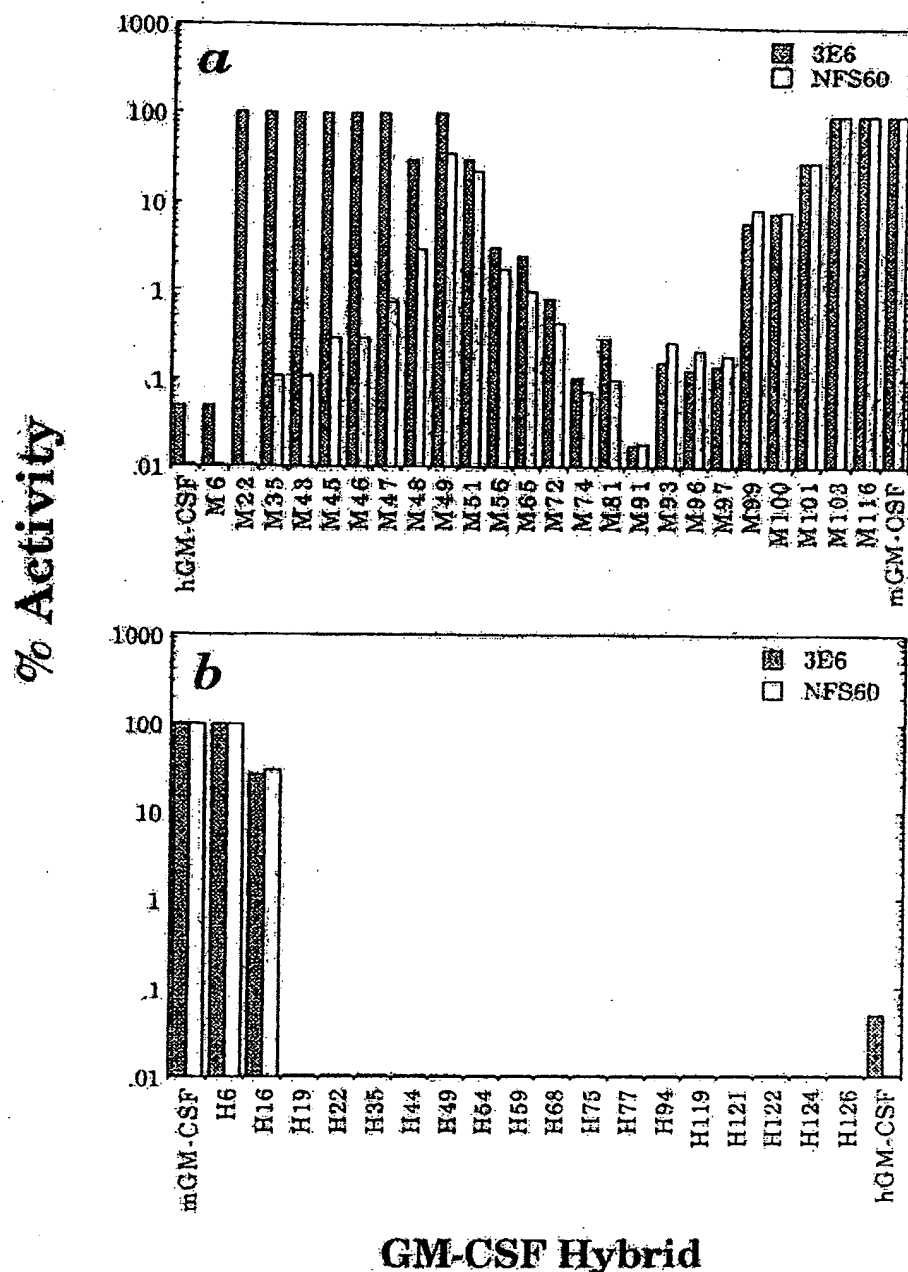


Fig. 1. Hybrid GM-CSF proteins. **a**, Amino acid sequences of human and mouse GM-CSF. Sequences are aligned for optimal homology. **b**, Pictorial representation of the hybrid GM-CSF proteins. Open bars correspond to hGM-CSF sequences, and shaded to mGM-CSF. The naming of the hybrid proteins follows the convention that either an M (mouse) or H (human) designates the species of origin of the N-terminal portion of the hybrid, where the number following indicates the amino acid at which the two polypeptide segments are fused.

carboxy-terminal 22 amino acids of mGM-CSF could be replaced with the corresponding human residues without affecting biological activity (Shanafelt *et al.*, 1991). Both 3E6 and NFS60 fully respond when activated with the hybrids M103 and M116, suggesting that these hybrids can bind with high affinity to mGM-R α and mGM-R β . Hybrid ligands M56 to M101 show a reduced activity with both 3E6 and NFS60. We have no explanation for this reduction. However, most importantly, both NFS60 and 3E6 have an equivalent response to these hybrids. With hybrids M49 to M22 the responses of 3E6 and NFS60 are different. Whereas there is a minimal response of these hybrids on NFS60 cells, there is full biological activity to hybrids M49–M22 on 3E6 cells. Since the only difference between NFS60 and 3E6 is

the presence of hGM-R α , these hybrids seem to be interacting with this receptor in combination with the mGM-R β receptor to form high affinity binding sites capable of transducing the biological response. This is most clearly demonstrated with hybrid M22: M22 shows full biological activity on 3E6, yet it is completely inactive on NFS60. Apparently, the presence of only 22 N-terminal amino acids of mGM-CSF on hGM-CSF is sufficient to ensure high affinity binding. Replacement of additional mouse residues in hybrid M6 eliminates this ability; its activity is identical to hGM-CSF. This series of experiments identifies amino acid residues 7–22 as the region in mGM-CSF responsible for specific interaction with mGM-R β .

To examine the effect of these hybrids in the absence of



GM-CSF Hybrid

Fig. 2. Relative activity of GM-CSF hybrid proteins on NFS60 and 3E6 cells (NFS60 stably transfected with hGM-R α). Results are expressed as % activity relative to that of wild-type mGM-CSF (see Materials and methods); hybrid proteins exhibiting > 100% activity (M22, M35 and M43 to M47 on 3E6) are shown with activity equal to 100%. These particular hybrid proteins probably show greater than wild-type activity because of the higher expression of hGM-R α compared with mGM-R α on 3E6 (~10-fold). All active hybrid proteins (except M6, which appeared identical to hGM-CSF) had a maximal plateau response equivalent to mGM-CSF on 3E6. Open bars represent the response of NFS60 and shaded bars the response of 3E6. **a**, Response to amino-terminal mouse-carboxy-terminal human GM-CSF hybrids (M series). **b**, Response to amino-terminal human-carboxy-terminal mouse GM-CSF hybrids (H series).

mGM-R α , we used an IL-2 dependent mouse T cell line that was stably transfected with hGM-R α and mGM-R β [CTLL(h α -m β)] (Kitamura *et al.*, 1991a). This cell line does not express any endogenous GM-CSF receptor subunits. Its response to the hybrids was identical to that of 3E6 with two exceptions: mGM-CSF, as expected, is inactive on the CTLL line, since no mGM-R α is present; for the same reason, hybrids M103 and M116 which were active on 3E6, are now inactive (data not shown).

We have also described the response of NFS60 to a collection of amino-terminal human-carboxy-terminal mouse GM-CSF hybrids (H series) (Shanafelt *et al.*, 1991).

Figure 2b shows the response of this series on 3E6 as compared with NFS60. Only two hybrids show activity, H6 (100%) and H16 (~30%). All other hybrids were inactive (<0.01%). Having determined the region of interaction of mGM-CSF with mGM-R β as residues 7–22, this result can now be understood. Only hybrids H6 and H16 still have an mGM-R β binding domain. This narrows down the number of residues of mGM-CSF critical for this interaction to amino acids 17–22. This region coincides precisely with that determined by scanning deletion analysis to be critical for biological activity (Shanafelt and Kastelein, 1989). Structurally this region is predicted to be part of an amino-

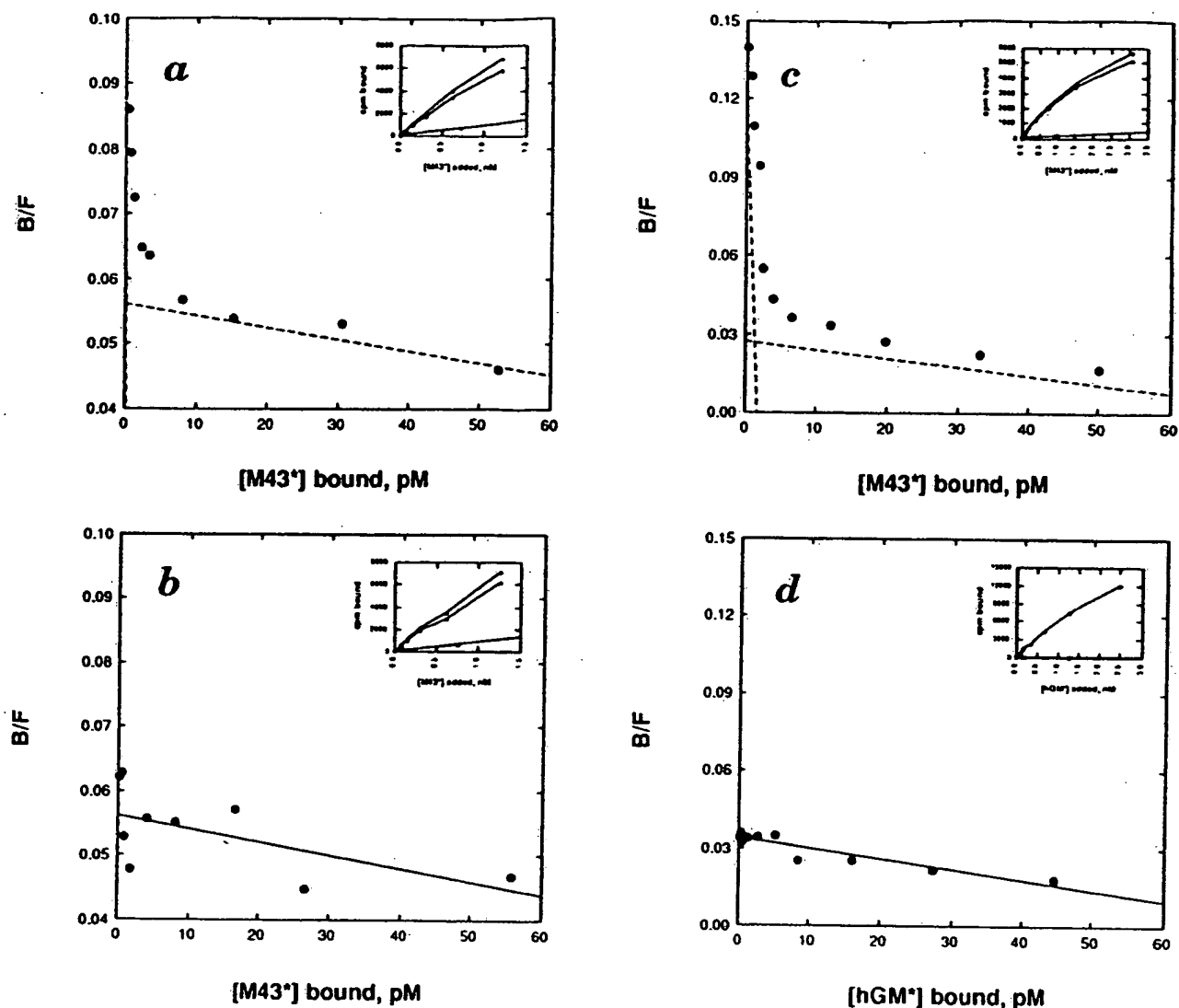


Fig. 3. Scatchard analysis of M43 and hGM-CSF binding to 3E6 and CTLL(h α -m β) cells. Solid lines (single binding site) and dashed lines (two binding sites) represent best fit values obtained from the LIGAND program (Munson, 1983). Each point represents the mean of at least three sets of data. Insets display the equilibrium binding isotherm (total bound, specifically bound, and non-specifically bound [125 I]M43 or [125 I]hGM) for the respective Scatchard plot. a, [125 I]M43 binding to 3E6; b, [125 I]M43 + 500 nM unlabeled mGM-CSF binding to 3E6; c, [125 I]M43 binding to CTLL(h α -m β); d, [125 I]hGM binding to CTLL(h α -m β).

terminal α -helix stretching from residue 15 to 27 (Parry *et al.*, 1988). Our evidence suggests that this region functions as an α -helix, since proline substitutions destroy biological activity (Altmann *et al.*, 1991; A.B. Shanafelt and R.A. Kastelein, unpublished data).

Characterization of binding of M43

The biological response elicited by the active hybrids is most probably due to the formation of high affinity GM-CSF receptor complexes. To show the presence of high affinity sites, one of the fully active hybrids, M43, was purified, radio-labeled with 125 I, and used in binding experiments on 3E6 and CTLL(h α -m β) (Figure 3). 3E6 expresses two classes of receptor for this ligand; these classes have differing affinities (Figure 3a; Table I). As expected, the M43 high affinity sites could be completely competed by mGM-CSF, since 3E6 still has the ability to form high affinity binding sites with mGM-R α and -R β (Figure 3b). Scatchard analysis of M43 binding to CTLL(h α -m β) also reveals high and

Table I. Equilibrium dissociation constants and binding site values for 3E6 and CTLL(h α -m β)

Ligand	K_d^a	Binding sites/cell ^b
3E6		
[125 I]M43	13 pM	59
	5.6 nM	38 000
[125 I]M43 + 500 nM mGM-CSF	4.8 nM	33 000
CTLL(h α -m β)		
[125 I]M43	15 pM	2 200
	3.1 nM	100 000
[125 I]hGM-CSF	2.5 nM	100 000

^aMean values calculated from the LIGAND program (Munson, 1983).

low affinity binding sites with K_d s of 15 pM and 3.1 nM, respectively (Figure 3c; Table I). High affinity binding sites on CTLL(h α -m β) are not seen with hGM-CSF as ligand (Figure 3d).

Comparison of the amino acid sequences of mouse and human GM-CSF in the proposed β receptor binding region shows only two differences: Glu17 and Lys20 in mGM-CSF versus Asn17 and Gln20 in hGM-CSF (Figure 1a). Random substitution of Glu17 in mGM-CSF has only a modest effect on biological activity, indicating limited involvement of this residue in the interaction (data not shown). Strong evidence for the direct involvement of residue 20 was obtained when we replaced Gln20 in hGM-CSF by Lys, the mGM-CSF residue found at position 20. Whereas hGM-CSF elicits a minimal response with the 3E6 or the CTLL(h α -m β) line, the hGM-CSF/Lys20 mutant has increased biological activity in both plateau and half-maximal response (Figure 4). Although this result identifies Lys20 as an important residue involved in the interaction with the mGM-R β , it is evident that other residues must also contribute; the hGM-CSF/Lys20 mutant is still ~30-fold lower in activity than mGM-CSF (Figure 4a).

mIL-5 interaction with mGM-R β

It has been suggested that in the mouse, the same β receptor subunit is present in the high affinity complexes that bind IL-5 and GM-CSF (Devos *et al.*, 1991; Kitamura *et al.*, 1991a). High affinity binding of these cytokines requires the interaction of each ligand with a unique ligand binding α subunit and the shared β subunit. We expected that, if mIL-5 forms high affinity binding sites with the mIL-5-R α and mGM-R β , it would do so by using the same binding motif as mGM-CSF for binding to mGM-R β . We tested this possibility by generating a hybrid that consisted of amino-terminal mIL-5 and carboxy-terminal hGM-CSF residues. Specifically, we replaced amino acids 4–32 of hGM-CSF by amino acids 5–29 of mIL-5. This region of mIL-5 includes a predicted amino-terminal α -helix from residue 7 to 22 (Parry *et al.*, 1988). When this hybrid was tested on 3E6 it elicited a full biological response (Figure 4a). A similarly strong response was observed with the CTLL(h α -m β) line (Figure 4b). When CTLL cells were used that expressed the mGM-R β homologue AIC2A together with hGM-R α (Kitamura *et al.*, 1991a) instead of the mGM-R β subunit, no response of this hybrid was observed (data not shown). These results show that mIL-5 can use the same β receptor subunit as mGM-CSF. They also strongly suggest that receptor recognition by IL-5 follows the same principle underlying the mGM-CSF-mGM-R β interaction, i.e. the presentation of a few specific residues in the context of an α -helix. Sequence comparison shows that Lys20 and Glu21 in mGM-CSF are conserved in the proposed first helix of mIL-5 (Lys10 and Glu11; Table II). Moreover, the same pair of amino acids is generated in hGM-CSF when Gln20 is changed to Lys20, suggesting that besides Lys20, Glu21 may also be required for specific interaction with the mGM-R β subunit.

Discussion

Binding of GM-CSF to its functional receptor is a complex event that includes interaction with multiple receptor components. GM-CSF is able to bind with low affinity to the GM-R α subunit (Gearing *et al.*, 1989). The GM-R β subunit does not by itself bind GM-CSF, but in conjunction with the GM-R α subunit it forms the high affinity receptor. For a biological response to occur, GM-CSF has to interact

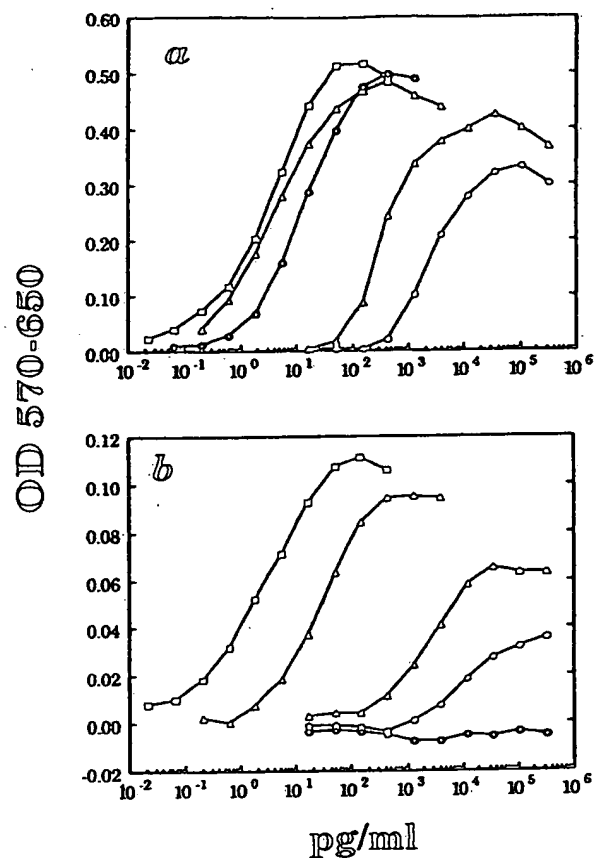


Fig. 4. Growth response of 3E6 and CTLL(h α -m β) cell lines. a, response with 3E6 and b, response with CTLL(h α -m β). Cells were incubated for 24 h in the presence of decreasing concentrations of mGM-CSF (○), hGM-CSF (□), M43 (□), mIL5-hGM-CSF hybrid (Δ), and hGM-CSF/Lys20 (△).

with both the GM-R α and the GM-R β receptor (Hayashida *et al.*, 1990). The work presented here shows that a small, discrete region in mGM-CSF is responsible for the interaction with the GM-R β . Structurally, this region is most likely presented to the β -receptor as an α -helix (Bazan, 1990a; Parry *et al.*, 1988) with at least Lys20 directly interacting with the receptor. Evidence for the existence of the same binding motif has recently been described for the IL-2 receptor system. Exhaustive mutational analysis of mIL-2 led to the identification of Asp34 as the only residue interacting directly with the IL-2-R β component (Zurawski *et al.*, 1990; Zurawski and Zurawski, 1989). The only other requirement for this interaction to occur was the presentation of Asp34 in the context of an α -helix. The similarities between this receptor system and the one described in this paper are remarkable. In both cases the interaction involves residue(s) in the amino-terminal α -helix of the cytokine and is targeted to a receptor component that by itself binds the ligand poorly (in the case of IL-2, Ringheim *et al.*, 1991) or not at all (in the case of GM-CSF, Gorman *et al.*, 1990; Hayashida *et al.*, 1990). A third example is the interaction of mIL-5 and mGM-R β . This is a biologically relevant interaction, since the GM-R β subunit seems to be required for high affinity IL-5 binding. Although we have not yet precisely identified the responsible residues within the mIL-5 amino-terminal helix, it is likely, based on the work presented here, that the interaction between IL-5 and its receptor follows the same general pattern.

Table II. Amino acid homology in predicted amino-terminal helices of cytokines

Cytokine	Proposed helix	Amino acid sequence ^a															
mIL-2 ^b	31-39			L	L	M	D	L	Q	E	L	L					
hIL-2 ^b	17-25			I	V	K	D	L	Q	M	I	L					
mIL-3	19-28		S	I	V	K	E	I	I	G	K	L					
hIL-3 ^b	18-27		N	M	I	D	E	I	I	T	H	L					
mIL-4	8-17		N	H	L	R	E	I	I	G	I	L					
hIL-4	8-17		I	T	L	Q	E	I	I	K	T	L					
mIL-5 ^b	7-22		T	V	V	K	E	T	L	T	Q	L	S	A	H	R	A
hIL-5	9-24		A	L	V	K	E	T	L	A	L	L	S	T	H	R	L
mIL-6	18-43		H	V	L	W	E	-	I	V	E	M	R	K	E		
hIL-6 ^b	18-43		R	Y	I	L	D	G	I	S	A	L	R	K			
mIL-7	9-20		G	K	A	Y	E	S	V	L	M	I	S	I			
hIL-7	9-20		G	D	Q	Y	E	S	V	L	M	V	S	I			
mIL-9	7-22		W	G	I	R	D	T	N	Y	L	I	E	N	L	K	D
hIL-9	7-22		A	G	I	L	D	I	N	F	L	I	N	K	M	Q	D
mIL-10	21-31		H	M	L	L	E	L	R	T	A	F	S				
hIL-10	21-31		N	M	L	R	D	L	R	D	A	F	S				
vIL-10	9-19		Q	M	L	R	D	L	R	D	A	F	S				
hEPO ^b	4-28	Y	L	L	E	A	K	E	A	E	N	I	T	G			
mG-CSF	19-30	F	L	L	K	S	L	E	Q	V	R	K	I				
hG-CSF ^b	13-24	F	L	L	K	C	L	E	Q	V	R	K	I				
mGM-CSF	15-28	H	V	E	A	I	K	E	A	L	N	L	L	D	D		
hGM-CSF ^b	15-28	H	V	N	A	I	Q	E	A	R	R	L	L	N	L		

^aOnly the pertinent portion of each helix is shown.

^bPredicted helices are from: for IL-2, Brandhuber *et al.* (1987) and Zurawski and Zurawski (1989); for hIL-3, Parry *et al.* (1988); for mIL-5, Parry *et al.* (1988); for hIL-6, Bazan (1990a); for hG-CSF, Parry *et al.* (1988); for hEPO, Bazan (1990a); for hGM-CSF, Parry *et al.* (1988). The location of the N-terminal helix in the other cytokines was based on comparable motifs from these secondary structure predictions.

The overall structural conservation of cytokines as four α -helical bundle proteins (Parry *et al.*, 1988) and the common receptor-cytokine binding motif described above hints at the existence of an amino-terminal recognition helix in many cytokines that exist as four α -helical bundle proteins. This recognition helix contains the primary and possibly the only specific site of interaction of the cytokine with one component of its functional receptor. In Table II, the sequences of proposed amino-terminal helices of a large number of cytokines are compared. There is an absolute conservation of a negatively charged residue (Glu/Asp) in each helix. Two other positions are conserved in relation to the negatively charged residue; if the charged residue is designated as position 0, residues at positions -2 and +5 are both hydrophobic. Upon examination of the spatial relationships in the helix, it is evident that the hydrophobic residues are located on one side of the helix separated by two helical turns, with the Glu/Asp residue in between these residues on the opposite side of the helix. While these conserved residues may define a core motif, other residues in the helix, such as Lys20 in mGM-CSF, possibly confer specificity to the interaction.

What residues of a cytokine receptor are targeted to interact with the proposed cytokine recognition helix? Cytokine receptors form a family of proteins with a number of conserved features (Bazan, 1990a,b). It is possible that one of these, the 'WSXWS' amino acid sequence in the extracellular domain near the transmembrane region, is the target region for this interaction. Recently, this region was implicated in a direct interaction between human growth hormone and the prolactin receptor, a member of this receptor family (Cunningham *et al.*, 1990). The binding was mediated by Zn²⁺ coordination of three residues in growth hormone, two of which are located in the amino-terminal helix of the protein, and a His residue in the immediate proximity of the 'WSXWS' sequence of the prolactin receptor. If the binding motif described here is directed

towards a conserved feature of a receptor subunit, e.g. the 'WSXWS' motif, then this principle may not be restricted to cytokine-R β interaction alone. For example, both GM-R α and -R β belong to this receptor family; therefore, it is possible that the same binding motif that governs interaction of GM-CSF and GM-R β also determines the interaction of GM-CSF with GM-R α . There is some evidence which suggests that this is indeed the case. Critical regions identified in mGM-CSF coincide with predicted α -helices, emphasizing the importance of these regions (Shanafelt and Kastelein, 1989). Interestingly, inspection of the sequence of human and mouse GM-CSF, as well as of IL-5, shows that in the carboxy-terminal helix of these molecules the proposed core binding motif is present. This C-terminal region of IL-5 has been shown to affect both its biological activity and receptor binding characteristics (McKenzie *et al.*, 1991).

A key question remains. GM-CSF has no measurable affinity for the β subunit without GM-R α , yet the equilibrium constant of the high affinity complex is in the picomolar range. It is likely that we have determined the specific ligand-receptor interaction that turns this complex into a high affinity receptor. However, other protein-protein associations must exist to account for this high affinity binding constant. It is not yet known how great the contribution of receptor-receptor interactions is to this process. The homology among cytokine receptors opens the interesting possibility that new receptor combinations could be formed that respond to appropriate hybrid ligands. Such hybrid ligands may be useful as antagonists since their potential to form high affinity complexes could be used to effectively compete for high affinity binding of the native ligand.

Materials and methods

Bacterial host strains and vectors

The *E. coli* K12 strain JM101 (Messing, 1983) was used as host for the propagation and maintenance of M13 DNA. CJ236 (Kunkel *et al.*, 1987)

was used to prepare uracil DNA for use in site-directed mutagenesis. AB1899 (Howard-Flanders *et al.*, 1964) was used as the host for expression of wild-type and mutant human and mouse GM-CSF proteins. Either pINIIIomPH3 (Lundell *et al.*, 1990) or pOMPTH3 (a tetracycline resistant variant of pINIIIomPH3) was used as the expression vector for all GM-CSF genes. Elsewhere, we have described the expression of biologically active, mature GM-CSF with this *E. coli* secretory expression system (Greenberg *et al.*, 1988).

Mutagenesis, recombinant DNA, and sequencing protocols

Site-directed mutagenesis followed the protocol described by Kunkel *et al.* (1987). Individual clones were sequenced using the dideoxynucleotide method (Sanger *et al.*, 1977) with modifications described in the Sequenase (United States Biochemical) protocol. M13 (replicative form) DNA containing correct mutations was cleaved with *Xba*I and *Bam*HI (New England Biolabs) for cloning into pINIIIomPH3. Synthetic oligonucleotide overhangs corresponding to amino acid residues 5–29 of mL-5 were ligated into the synthetic hGM-CSF gene (Shanafelt *et al.*, 1991) cleaved with *Bgl*II and *Sac*II. This construct replaced amino acid residues 5–32 of hGM-CSF with those of mL-5 in the pINIIIomPH3 expression vector. The generation of the human–mouse GM-CSF hybrids is described elsewhere (Shanafelt *et al.*, 1991).

Preparation and quantification of protein extracts

Expression and quantification of mutant proteins is described elsewhere (Shanafelt *et al.*, 1991). Briefly, all mutant proteins were produced in *E. coli* AB1899 and periplasmic extracts were prepared by osmotic shock (Shanafelt and Kastelein, 1989). When purified, protein prepared by osmotic shock behaved identically to the pure product (A.B. Shanafelt and R.A. Kastelein, unpublished results); the specific activity of osmotic shock extracts varied by <10% for a given mutant in multiple assays performed over several months time. The amount of mutant polypeptide produced was determined using quantitative immuno-slot blotting. The error in the calculated concentration of GM-CSF protein by this method was estimated to be 2-fold based on repetitive protein samples.

Transfection of mammalian cell lines

The low affinity hGM-CSF receptor (Gearing *et al.*, 1989) was stably transfected to the mGM-CSF dependent cell line NFS60 with the Lipofectin reagent (BRL) using the manufacturer's suggested protocol. The neomycin resistance gene was used as the selection marker. Stable transfectants were selected with G418 at 1 mg/ml; the clone used in this study had the strongest response to hGM-CSF and was designated 3E6. The generation of the CTLL(α –m β) cell line has been previously described (Kitamura *et al.*, 1991a).

Proliferation assays for human and mouse GM-CSF activity

Protein extracts were assayed using the mouse GM-CSF dependent myeloid leukemia cell line NFS60, 3E6 and CTLL(α –m β). Sample concentrations were adjusted to 108 000 pg/ml and titrated in quadruplicate to 1.8 pg/ml. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay described by Mosmann (1983) was used to measure the extent of proliferation, and absorbance values were read with a V_{max} kinetic microplate reader (Molecular Devices). The concentration of each mutant and wild-type GM-CSF that gave 50% maximum response was determined, and specific relative activity was calculated using the relationship:

$$\% \text{ Activity} = ([\text{wild-type}]_{50}/[\text{mutant}]_{50}) \times 100\%$$

where $[\text{wild-type}]_{50}$ and $[\text{mutant}]_{50}$ are the concentrations of wild-type and mutant GM-CSF proteins, respectively, that gave 50% maximum response in the NFS60 or 3E6 assays.

Purification and binding characterization of M43

M43, expressed in *E. coli*, was purified to homogeneity from periplasmic extracts by gel filtration through Superdex-75 (Pharmacia-LKB) followed by affinity chromatography using agarose-coupled (AminoLink, Pierce Chemical Co.) anti-mGM-CSF MAb 35E10 (kindly provided by M. Pearce). Purified M43 was radiolabeled with ^{125}I using the Bolton and Hunter reagent (ICN). Binding assays were performed as follows: 3E6 cells (maintained in mGM-CSF) were harvested and incubated with 1 ml ice cold 10 mM NaPO_4 , 150 mM NaCl , pH 3.0 for 2 min, diluted to 50 ml with 10 mM NaPO_4 , 150 mM NaCl , pH 7.0, centrifuged and finally resuspended in $1 \times$ Hanks' balanced salts solution (Gibco/BRL) containing 0.1% BSA, 0.02% NaN_3 and 10 mM HEPES, pH 7.5 (HBAH buffer). CTLL(α –m β) cells (maintained in mL-2) were prepared without acid treatment. 1×10^6 (3E6) or 1×10^5 (CTLL(α –m β)) cells in 200 μl HBAH

buffer were incubated with decreasing concentrations of ^{125}I hGM-CSF (Amersham, sp. act. 947 Ci/mmol) or ^{125}I M43 (sp. act. 400 Ci/mmol) at 4°C with continuous agitation for 4 h. Non-specific binding was determined by including unlabeled hGM-CSF or M43 as appropriate at a concentration of 1 μM . Cell bound radioactivity was separated from free ligand by centrifugation at 4°C (4 min, 12 000 g) through diethyl-phthalate:dibutylphthalate (2:3), and bound and total radioactivity were measured with a Cobra 5010 γ -counter (Packard). The equilibrium binding data were analyzed using the LIGAND program (Munson, 1983).

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References

- Altman, S.W., Johnson, G.D. and Prystowsky, M.B. (1991) *J. Biol. Chem.*, **266**, 5333–5341.
- Bazan, J.F. (1990a) *Immunol. Today*, **11**, 350–354.
- Bazan, J.F. (1990b) *Proc. Natl. Acad. Sci. USA*, **87**, 6934–6938.
- Brandhuber, B.J., Boone, T., Kenney, W.C. and McKay, D.B. (1987) *Science*, **238**, 1707–1709.
- Budel, L.M., Elbaz, O., Hoogerbrugge, H., Delwel, R., Mahmoud, L.A., Lowenberg, B. and Touw, I.P. (1990) *Blood*, **75**, 1439–1445.
- Chiba, S., Shibuya, K., Piao, Y.-F., Tojo, A., Sasaki, N., Matsuki, S., Miyagawa, K., Miyazono, K. and Takaku, F. (1990) *Cell Regul.*, **1**, 327–335.
- Cunningham, B.C., Bass, S., Fuh, G. and Wells, J.A. (1990) *Science*, **250**, 1709–1712.
- Devos, R., Plaetinck, G., Van der Heyden, J., Cornelis, S., Vandekerckhove, J., Fiers, W. and Tavernier, J. (1991) *EMBO J.*, **10**, 2133–2137.
- Gearing, D.P., King, J.A., Gough, N.M. and Nicola, N.A. (1989) *EMBO J.*, **8**, 3667–3676.
- Gorman, D.M., Itoh, N., Kitamura, T., Schreurs, J., Yonchara, S., Yahara, I., Arai, K. and Miyajima, A. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 5459–5463.
- Gough, N.M. and Nicola, N.A. (1989) In Dexter, T.M., Garland, J.M. and Testa, N.G. (eds), *Colony-Stimulating Factors: Molecular and Cellular Biology*. Dekker, New York, pp. 111–153.
- Greenberg, R. *et al.* (1988) *Curr. Microbiol.*, **17**, 321–332.
- Hayashida, K., Kitamura, T., Gorman, D.M., Arai, K., Yokota, T. and Miyajima, A. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 9655–9659.
- Howard-Flanders, P., Simson, E. and Theriot, L. (1964) *Genetics*, **49**, 237–246.
- Itoh, N., Yonchara, S., Schreurs, J., Gorman, D.M., Maruyama, K., Ishii, A., Yahara, I., Arai, K. and Miyajima, A. (1990) *Science*, **247**, 324–327.
- Kitamura, T., Hayashida, K., Sakamaki, K., Yokota, T., Arai, K. and Miyajima, A. (1991a) *Proc. Natl. Acad. Sci. USA*, **88**, 5082–5086.
- Kitamura, T., Sato, N., Arai, K. and Miyajima, A. (1991b) *Cell*, **66**, 1165–1174.
- Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987) *Methods Enzymol.*, **154**, 367–382.
- Lopez, A.F., Eglinton, J.M., Lyons, A.B., Tapley, P.M., To, L.B., Park, L.S., Clarke, S.C. and Vadas, M.A. (1990) *J. Cell. Physiol.*, **145**, 69–77.
- Lundell, D. *et al.* (1990) *J. Indust. Microbiol.*, **5**, 215–228.
- McKenzie, A.N.J., Barry, S.C., Strath, M. and Sanderson, C.J. (1991) *EMBO J.*, **10**, 1193–1199.
- Messing, J. (1983) *Methods Enzymol.*, **101**, 20–78.
- Metcalfe, D. (1986) *Blood*, **67**, 257–267.
- Mosmann, T. (1983) *J. Immunol. Methods*, **65**, 55–63.
- Munson, P.J. (1983) *Methods Enzymol.*, **92**, 543–576.
- Park, L.S., Friend, D., Price, V., Anderson, D., Singer, J., Prickett, K. and Urdal, D.L. (1989a) *J. Biol. Chem.*, **264**, 5420–5427.
- Park, L.S. *et al.* (1989b) *Blood*, **74**, 56–65.
- Parry, D.A., Minasian, E. and Leach, S.J. (1988) *J. Mol. Recognit.*, **1**, 107–110.
- Ringheim, B.E., Freimark, B.D. and Robb, R.J. (1991) *Lymphok. Cytok. Res.*, **10**, 219–224.
- Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
- Schreurs, J., Hung, P., May, W.S., Arai, K. and Miyajima, A. (1991) *Int. Immunol.*, in press.

- Shanafelt, A.B. and Kastelein, R.A. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 4872-4876.
- Shanafelt, A.B., Johnson, K.E. and Kastelein, R.A. (1991) *J. Biol. Chem.*, **266**, 13804-13810.
- Zurawski, S.M. and Zurawski, G. (1989) *EMBO J.*, **8**, 2583-2590.
- Zurawski, S.M., Imler, J.L. and Zurawski, G. (1990) *EMBO J.*, **9**, 3899-3905.

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